Identification and synthesis of semiochemicals from arthropods

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1. Introduction

Arthropods which include Chelicerata (scorpions, crabs, spiders, ticks, mites and others), Crustacea (crabs, lobsters, shrimps, barnacles and woodlice), Uniramia (insects, centipedes, millipedes), and the extinct Trilobita (extinct trilobites) are by far the most successful phylum of animals, both in diversity of distribution and in number of species and individuals. They have adapted successfully to life in water, on land, and in the air. These arthropods can move actively in search of food, shelter, potential mates, or to escape from predators, and sometimes are even passively carried via air or water currents. The environment in which the organism is in determines to a certain extent the suitable cue that is able to guide its movement along with influence of the target. It is the property of the environment, as well as those of the target itself, that determines the sensory modality used in a particular oriented behavior, since arthropods are designed with good sensory systems for exploiting information of every possible sensory modality.[1]

All the above mentioned activities are considered as essential for life. To fulfill them, these organisms have to adapt a medium which follows either a physical pathway or a chemical pathway.[2] Regarding this phenomena the arthropods have developed several communication channels like optical, acoustical and tactile modes which are integrated into their life style. The communication via information bearing compounds, or semiochemicals, is another very powerful sensory channel especially exploited by small arthropods living in a spacious environment.

Semiochemicals are message bearing compounds that mediate interactions between various organisms in the form of communication signals. Many of the semiochemicals are volatile and are perceived through olfaction. Depending on the species within which these messages are being transferred i.e. interspecific or intraspecific interactions, the semiochemicals are further classified into allelochemicals and pheromones.[3] Allelochemicals are those compounds which operate interspecifically whereas pheromones mediate intraspecific interactions. Based on the favorability of the interaction allelochemicals are further divided into allomones which are released by an individual (emitter) to induce behavioral responses in other individuals (receiver) in a manner beneficiary to the emitter, e.g. defensive secretions, kairomones which are adaptively favorable to the receiver, e.g. attractant odors, and synomones which are advantageous to both, the emitter and the receiver, e.g. sex

pheromones.[2] Unlike hormones which are released inside the body, pheromones can be termed as ectohormones (released outside the body of the insect or animal) and can be defined as the chemical compounds which are released by one individual to trigger specific responses from other individuals of the same species.[4] Depending on the type of specific reaction which can be behavioral or physiological they can be divided into two major categories

I) Primer pheromones – these are the compounds which help coordinate the timing of physiological development. Examples of this class include stimulation of sperm production in fish or termite caste determination.[5]

II) Releaser pheromones – compounds which upon release have immediate effects on the behavior of the receiver.[5]

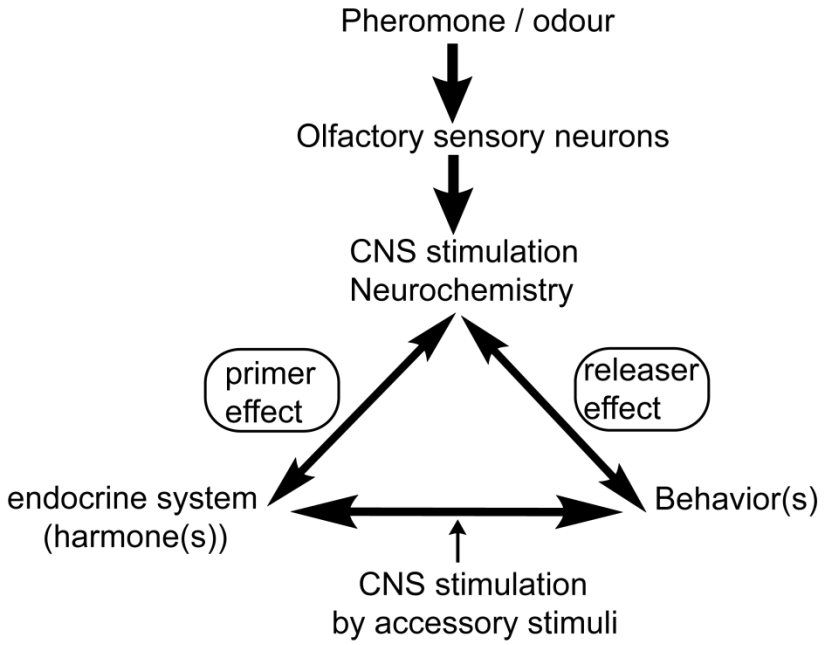


Figure.1 Pheromones can be a stimulus leading to a prompt behavioral response by nerve impulses from brain (releaser effect) or can act indirectly by stimulation of hormone secretion resulting in physiological changes (primer effect).

On the basis of the interaction mediated, primer pheromones are further subdivided into several following categories.[6]

1. Aggregation pheromones – increase the density of conspecifics (males as well as females) which might function in defense against predators, mate selection, or overcoming the host resistance by mass attack.

2. Alarm pheromones – serve in causing the dispersion (as an alert signal) or aggressive behavior in a group of individuals as a response to predation.[7,8]
3. Trail pheromones – common in social insects like ants who use these compounds as trail to mark their path. They can also guide the individuals to follow this path.[9]
4. Sex pheromones – compound or mixture of compounds that alter the behavior of one or both members of the mating pair, thereby regulating the different stages of the mating process.[2]
5. Territorial pheromones – some territorial mammals often release chemicals substances which are deposited on a portion of the home range that induces aversive or agonistic behavior in intruders belonging to the same species.[10]
6. Epideictic pheromones – also called spacing pheromones which are usually seen in insects. Commonly, female insects leave substances around the vicinity where they clutch their eggs so that other insects will automatically look for another place. This pheromone type is similar to the territorial pheromones that animals use to label the scope of their territory.

Pheromones can be further classified depending on the range of their activity i.e. the distance over which they can find or identify their conspecifics. The compounds which are volatile show long range activity and hence, can mediate long range interactions (even up to 11 km) as in emperor moth.[11] Those compounds which are non volatile operate on contact in short range interactions. Hydrocarbons ranging from 11 to 49 carbon atoms[12] predominating on the cuticle of arthropods[13] can serve as some examples for short and close range interactions.

These cuticular lipid layer comprising of normal and branched saturated and unsaturated hydrocarbons, free fatty acids, free alcohols, alkyl esters, glycerides, sterols and aldehydes[14,15] are efficiently used by solitary and social insects as recognition systems,[16] with which these insects are able to identify, differentiate its own species, sex or kin from that of other insects,[17,18] along with its main objective of preventing the insect from dehydration. Recent developments in studies related to insect cuticular compositions revealed that they are species-specific,[19,20] sex-specific[21,22,23] (attractants, aphrodisiacs, or inhibitors), in social insects colony and caste specific,[24] moisture barriers and sometimes are also chemical mimics in some

parasitic insects,[25] proving to be very important in the everyday activities of many insect species.

On the other hand, research on spider cuticle reported that it can also serve as a source for pheromones,[26] mediate intraspecific agonistic behavior,[27] or induce (female cuticular extracts) courtship behavior in males.[28] Other investigations performed on spider lipids disclosed that they are also essentially consisting of long chain aliphatic hydrocarbons which include normal and branched alkanes, primarily 2-methyl alkanes with even number of carbon atom chain being most abundant, along with smaller amounts of long chain aliphatic alcohols and aldehydes, fatty acids, glycerides, cholesterol and wax esters.[29] Although the cuticle of spider delivers a variety of compounds which might serve as candidates for species recognition, not much work on spider lipids has been performed to establish their significance in chemical communication.[30]

Cuticular ingredients or volatile components used in chemical communication (short range and long range interactions), can have very different structures. Understanding their biosynthetic origin will add a valuable sense to their versatility and helps to group them according to their origin. Semiochemicals in arthropods are biosynthetically acquired either from primary metabolites or formed as secondary metabolites. The latter ones are by far more common and are in the focus of pheromone research. These biosynthetic formations are accomplished by specific enzymes which catalyze a wide variety of reactions.

Biosynthesis involves building up of chemical compounds comprising of small molecules (building blocks). These processes are enzymatically driven and hence induce selectivity and specificity during the mechanisms and to the products as well.
Most of the hydrocarbon molecules comprising of branched, unbranched, saturated and unsaturated molecules are formed biosynthetically by decarboxylation of the appropriate fatty acids, which in turn are formed by condensation of acetyl or malonyl coenzyme A units.[31] The odd numbered fatty acids encountered in insects are biosynthesized starting from a propionate group to which are added the acetate units until the desired chain length is obtained. The same mechanism is used for the

formation of methyl branched fatty acids, using methylmalonyl instead of malonyl extender groups. [31]

In order to trace out the biosynthetic route that a particular molecule has followed, it is necessary to identify the ultimate source of primary metabolism from which the compound of interest derives, and also the intermediates through which a final product is formed. The former task is somewhat easy in comparison whereas the latter objective might prove difficult directing with false clues. The usual method of study is to suggest a possible precursor and to feed it to the biosynthesizing system by introducing isotopic labeling at some position in the precursor so that the sequence of reactions can be traced out. These isotopes can either be radioactive isotopes which can be followed by radiation or can be a stable heavy isotope which can be detected by mass spectrometry or NMR. Extensive studies employing these techniques have made the investigation of semiochemical biosynthesis in arthropods more convenient.

The following chapters of this thesis mainly focus on the work conducted on natural products from arthropods, which include extraction, analysis, structure elucidation, synthesis, bioassay, chiral studies, and tracing of biosynthesis which are important to get a clear concept concerning the Involvement of semiochemicals in chemical communication and try to contribute to the ongoing research in the field of chemical ecology.

2. Aim

Semiochemicals play an important role in nature to modify behavior of individuals and thus shape the environment. In the present study four projects have been performed. Two projects emphasize on spiders, since not much focus has been reported on their communication behavior so far. Additionally, pheromones in some agricultural important arthropods need to be identified to overcome pests.

Although much research that has been performed till date suggesting the widespread use of pheromones by spiders, not many have been structurally elucidated. The first project (chapter 3) concentrates on identification and synthesis of volatile extracts obtained from spider *Argiope bruennichi* along with bioactivity and chiral resolution studies.

Recent advances in spider semiochemicals suggested that cuticular lipid profiles of spiders are similar to that of arthropods with little variation compared to insects. To get a deeper perception, male and female cuticular lipid profiles of the spider *Argyrodes elevatus* were analyzed in this research group[32] and synthesis performed.[33] Stereoselective synthesis targeting these long chain methyl branched esters to identify the exact configuration and comparisons with the natural compounds is the major goal of this project (chapter 4).

The beetle *Pachnoda interrupta*, commonly called sorghum chafer, is a serious pest in the northern part of Ethiopia. Currently, there are no efficient control methods, except by mass trapping which would possibly reduce the population. Invention of some attractive lures might also prove beneficiary. Observations regarding the mating and aggregation behavior of the beetle in the field led us to suspect the existence of a pheromone which might possibly be responsible for this behavior. Analyzing the natural extracts from this beetle to figure out the possible attractants and to synthesize novel unsaturated hydrocarbons was the main intention of the project (chapter 5).

Females of the winter moth *Operophtera brumata* emit a long chain poly unsaturated hydrocarbon (3*Z*,6*Z*,9*Z*)-1,3,6,9 nonadecatetraene as pheromone.[34] In order to resolve the biosynthetic pathway of this pheromone, attempts were made to synthesize isotopically labeled polyunsaturated fatty acid and fatty acid methyl esters.

Introduction of stable heavy isotopes at any one end of the fatty acid chain and following investigations of the biosynthesis are the main intentions of this project (chapter 6).

3. Identification, synthesis and bioactivity of the pheromone released by the spider *Argiope bruennichi*

3.1 Introduction of spiders.

Spiders in common to many arthropods, exist in all ecological environments with an exception of air and sea. They are polyphagous carnivores (with few exceptions) mostly feeding on insects, but certain other arthropods such millipedes and sow bugs are consumed as well. Feeding behaviors of spiders vary markedly depending on spider toxin (venom), spider silk, or existence of cheliceral teeth (Figure 2). Like all other animals their behavior is controlled by the central nervous system[35] which include sensory organs like mechanoreceptors, chemoreceptors, and visual receptors out of which the mechanoreceptors are the most important.[36] The most characteristic feature of spiders is the ability to produce silken threads which is a secretary product of spinning glands (via extruded spinnerets). Spider silk which is a composite of α- chains and β-pleated sheets is proteinaceous in nature and belongs to fibroins.[37] During evolution, spiders have developed webs (or snares) from the silk which is quite an exceptional character. Furthermore they modified these webs into different varieties and shapes in order to enable the prey catching strategies.[38] The radii of these webs serve in several communication behaviors like prey capture or during courtship when the conspecifics are approaching. The webs are also supposed to carry messenger signals in the form of chemical molecules for pheromonal activity or even might defend them against predators.

The wandering and sedentary spiders both have developed diversified moving and prey capturing habits. They move by ballooning mechanism[39] which is by emitting a line of silk that is caught in the wind (locomotion on thread) or execute normal movement with legs. However not all spiders depend on the webs for their prey capturing process. There are some wandering spiders which gain their nutrition by hunting[40] or even by leading a parasitic life.

Female spiders are generally larger than males besides few exceptions. Due to their small body size males can move faster, mature earlier (since it requires fewer molts). Once males reach maturation they leave their retreats and become wanderers,

searching for the females, even sometimes no longer catching preys. For the reason of being dealt as a prey, the males cautiously approach females, along with a developed mating strategy. In some cases the males are eaten up by the females after or during mating, but this is not a common phenomenon. Males usually have a shorter lifespan whereas females live longer as they lay eggs and build cocoons after copulation. The males normally do not have any copulatory organs other than the pedipalps (Figure 2), transferring the sperm, an exceptional character in spiders.[35]

3.2 Taxonomy of spiders

Spiders belonging to the order *Araneae* consist of 100 different families with approximately 34,000 species. The *Araneae* are further classified into three suborders that include Mesothelae, Mygalomorphae, and Araneomorphae, basing on adaptive evolution (number, position, relative size, internal or external organs like eyes).

Mesothelae consists of 40 species in two genera or one family respectively which represent phylogenetically the oldest spiders known with primitive characteristic features. Mygalomorphae comprises over 2,200 species in 260 genera or 15 families. The majority of the spider species (around 90%) belong to the sub order Araneomorphae which contains 32,000 species in 2,700 genera or 90 families. The Araneomorphae are further divided into two systematic units, namely Palaeocribellatae and Neocribellatae. Neocribellatae consist two groups Austrochiloidea and Araneoclada out which the latter is the largest systematic unit which is again sub-divided into two small groups Haplogynae and Entelegynae.

The above mentioned natural classification at some point reflects the relationships among different species, although direct kinships among their community have not been yet proven. This systematic spider classification is just completely based on comparative morphology.[35]

3.3 Spider anatomy and function

Spiders unlike the insects have only two segments, prosoma (cephalothorax) and opisthosoma (abdomen), both of which are connected by a narrow stalk called pedicel that allows the multidirectional movement of the abdomen. The prosoma's essential functions include locomotion, food uptake and integration of nervous system. To enable the previous of activities, the prosoma serves as a place for the attachment of six pairs of appendages or extremities among which the first pair include chelicerae, the second pair include pedipalps and the remaining four pairs standing behind these two pairs serve as walking legs.

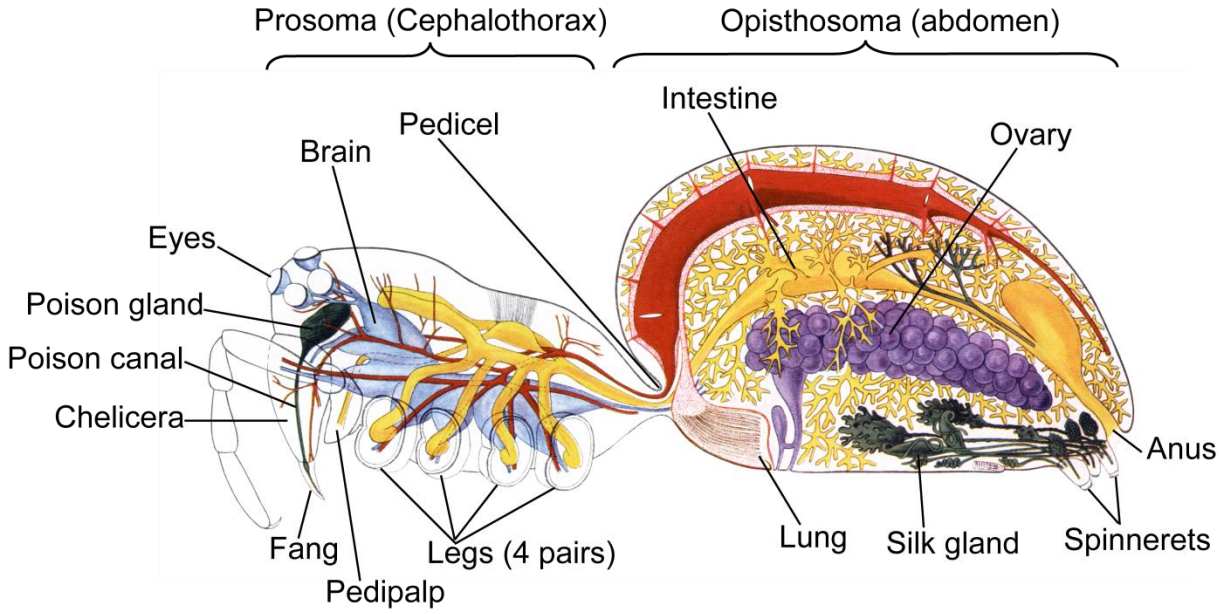


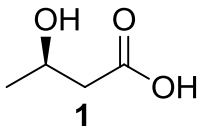
Figure.2 External and internal anatomy of spider (Photo: Benjamin Cummings)

The essential task of the chelicerae is to cause a bite, and along with fangs they involve in injecting venom into the prey when it is caught. In mature males spiders the pedipalps are modified into copulatory organs and are involved in transferring or releasing the sperms into the genital opening of the female during mating, which is quite an extraordinary aspect not observed in any other arthropods.[41] On the other hand, the opisthosoma chiefly performs vegetative tasks such as digestion, circulation, respiration, excretion, reproduction, and silk production. The abdomen has no appendages except for one to four (usually three) modified pairs of movable telescoping organs called spinnerets, which exclusively produce silk.

3.4 Previously identified spider pheromones

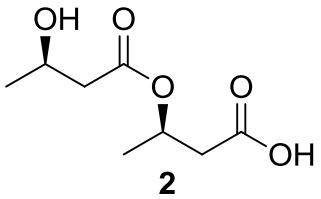
Recent advances in spider pheromones focus on pheromones emitted by females and received by males. Although this process in not universal,[42] it has been proposed that these pheromones are ubiquitous in nature, i.e. emitted both from cuticle and silk having a significant effect via airborne activity[43] or by coming into contact. Early experiments showed that they can also be heterospecific in nature.[44] Currently, sex pheromones have been conclusively identified in only six spider species: the sheet-web spider *Linyphia triangularis*,[45] the wandering spider *Cupiennius salei*,[46] the orb-web spider *Agelenopsis aperta,*[47] solitary spider *Tegenaria atrica,*[48] the Pholcid spider *Pholcus beijingensis*,[49] and the Australian Redback Spider *Latrodectus hasselti*.[50]

The first spider pheromone was identified in *Linyphia triangularis,* where the unmated females produce pheromones from the web to attract the males. Up on arrival the male spider starts to cut the threads of the web and rolls it into a round shaped ball, a phenomenon called web reduction. Males perform this activity to ensure no other conspecifics are attracted to interrupt the mating and enable a successful copulation with the first arrived male. This activity was never observed in the web of mated females suggesting the existence of an active compound on the unmated female webs, which on performing GC-MS analysis was identified to be a condensation product (dimer) of two molecules of (*R*)-3-hydroxybutyric acid (**1**) (Figure 3). Once on the silk, this dimer is unstable and decomposes slowly into (*R*)-3-hydroxybutyric acid (**1**) and further into crotonic acid. Based on several observations, a dual function for the dimer was assumed.



1

(*R*)-3-hydroxybutyric acid (HBA)



2

(*R,R*)-3-(3-hydroxybutyryloxy)-butyric acid (HBBA)

Figure.3 Sex pheromones of *Linyphia triangularis*

The dimer induces the web reduction behavior and reflects a propheromone because the degradation products (*R*)-3-hydroxybutyric acid and crotonic acid trigger male attraction. This pheromone system was also proven in the related species *L. tenuipalpis*, living in the same habitat, and other linyphiid species.[45] When applied heterospecifically, the responses were not so effective reflecting existence of species-specific chemicals on the web.

(*S*)-Dimethyl citrate (**3**) (Cupilure) was identified as the female sex pheromone (Figure 4) in the tropical wandering spider *Cupiennius salei*.[51] The pheromone is deposited on the female silk dragline which in turn serves as a signal post, resulting in the initiation of behavioral responses and other courtship traits in conspecifics males. The active compound was isolated from the silk of virgin females by solvent extraction using deuterated methanol and subsequent ^{1}H-NMR-analysis proved its structure. Synthesis and chiral resolution studies on GC confirmed the exact configuration of the natural compound. This compound was also identified from the silk extracts of closely related species *C.getazi* and *C.coccineus*.[52]

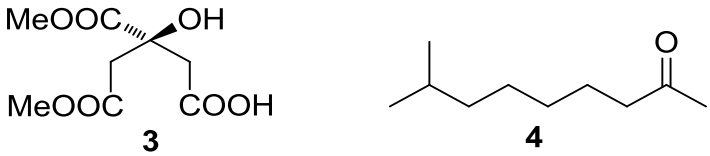


Figure 4. Spider sex pheromones **3** *Cupiennius salei;* **4** *Agelenopsis aperta*

Sexually mature virgin females of the desert spider *Agelenopsis aperta* release a volatile pheromone which was identified as 8-methyl-2-nonanone (**4**) (Figure 4), induce courtship responses in males. This compound was extracted from headspace extracts of virgin females two weeks after final molt and showed airborne activity. Bioassays with this compound attracted males and induced courtship behavior independent of the presence of a receptive female, in contrast to other behavioral traits. Doses of the pheromone in the range of ~500 ng were sufficient for the attraction of males in these experiments.

Females of *T. atrica* attach a contact sex pheromone to their web that consists of a complex mixture of methyl esters. Bioassays with synthetic substances showed that four fatty acids **5**-**8** and one methyl ester **9** (Figure 5) are particularly effective in eliciting sexual behavior in males.

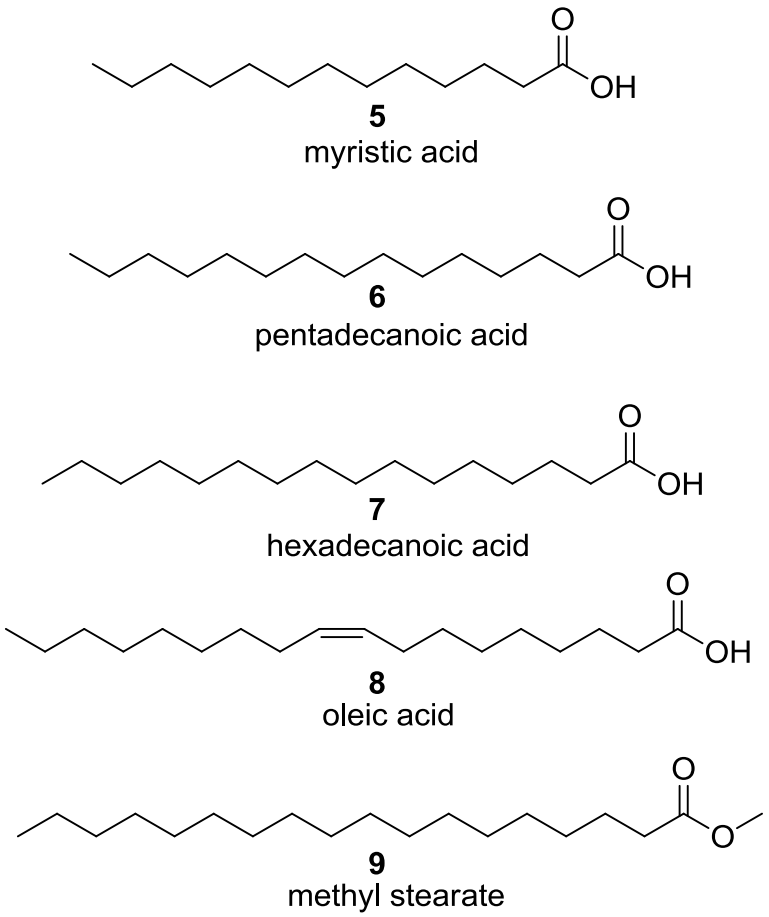


Figure 5. Sex pheromone system of spider *Tegenaria atrica*

The sex pheromone of *P. beijingensis* consists of a 2:1 ratio of (*E*,*E*)-farnesyl acetate (**10**) and hexadecyl acetate (**11**) (Figure 6), deposited on the web by sexually receptive females, the first multi-component pheromone found in spiders. Two-choice behavioral assays verified that the blend of (*E*,*E*)-farnesyl acetate (**10**) and hexadecyl acetate (**11**) in the ratio 2:1 attracted males at a dosage equivalent to the amounts of these compounds on spider web. Although both females and males of *P. beijingensis* can emit the pheromone, sexually receptive females release much more than males. Male *P. beijingensis* showed no attraction response to either FA or HA alone. However, males were not attracted when the pheromone dosage was much higher than that of a sexually receptive female's web.

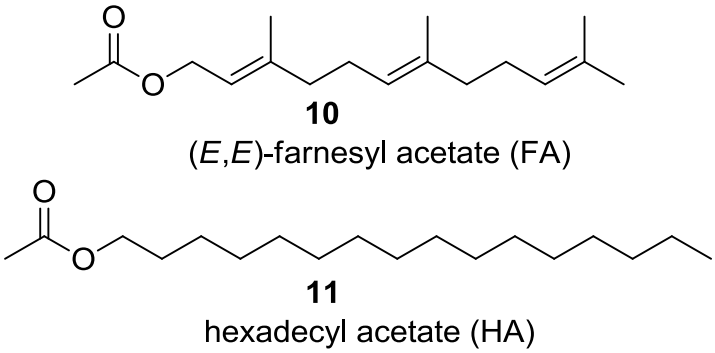


Figure 6. Sex pheromone system of spider *Pholcus beijingensis*

Analysis of silk from the unmated females of *L. hasselti* showed for the first time an amino acid derived sex pheromone, N-3-methylbutyryl-*O*-(*S*)-2-methylbutyryl-L-serine methyl ester (**12**) (Figure 7). Bioassay with synthetic sample triggered high level of activity when applied against males at a concentration of 100 µg. The activity was strongly dependent on stereochemistry as only (**L,*S*)-12** isomer attracted males while all the other isomers tested proved inactive indicating the wrong stereoisomer inhibits the positive response in males, the phenomenon not known in spiders before.

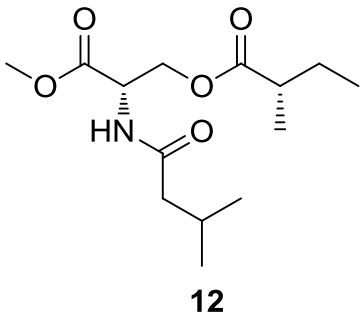


Figure 7. Sex pheromone of spider *Latrodectus hasselti*.

3.5 Life style of *Argiope bruennichi*

Orb weavers are one of the most successful spider families worldwide, with over 2,600 known species. Individuals of the genus *Argiope* (Araneidae) are quite distinguishable because of their characteristic weaving of orb shaped webs (Figure 8). Adult females of *Argiope bruennichi* have a shining cephalothorax which is covered with a silver colored hair, and a yellowish abdomen with white and black stripes across it. Adult females have a body length of 25 mm in contrast to males with a body length of 6 mm. When the females are loaded with eggs they can become enormous in size. Like all orb-weavers, they have ringed legs. The life history of *Argiope bruennichi* is characterized by ballooning[53] (aerial transport) and they prefer grassy or herbaceous vegetation in open, ephemeral or shrubby sites[54] in coarse-grained (patchy) landscapes.

Argiope bruennichi, commonly called as wasp spider builds its web very close to the ground (up to one meter above the ground). These spiders decorate their web with a prominent zigzag shaped silk that runs along the web threading which is known as stabilimentum featuring at the center of the orb web (Figure 8).

Figure 8. Female *Argiope bruennichi* in its orb shaped web

Although the exact function of this zigzag shaped white silk is uncertain, several ideas have been put forth saying it may be used to increase the spiders foraging efficiency[55] or to attract prey to the web by causing radiation of UV-light that is

sensitive to insects.[56] It might also involve in frightening the predators by shaking the web vigorously when something large is approaching that results in a blurry white spot. Some theories believe that it provides protection to the spider by either camouflaging it or making it appear larger.[57] Above all, the prey catching hypothesis[58,59] and predator-defense hypothesis[60] appear more reasonably logical and at the same time controversial.

When a prey is first caught in the web, *Argiope bruennichi* quickly immobilizes it by wrapping silk around the prey, and then injecting it with a paralyzing venom and a protein dissolving enzyme. In July the males mate with the females and often loose their life or some legs after copulation. The female makes a brown cocoon one month after mating and the young spiders hatch the next year in spring. The female dies in the winter. Earlier, this species was mainly distributed in the Mediterranean area but occurs also now in the temperate zone, probably triggered by climate changes.[61]

The existence of an airborne male attractant in a closely related species *Argiope trifasciata* was earlier reported.[62] Following chapters include the efforts made to investigate the chemical composition of male and female webs, headspace extracts and synthesis to conclude the pheromone molecule released by the female species of *Argiope bruennichi*.

3.6 Chemical composition of web extracts from *Argiope bruennichi* females

Webs of female virgin and mated *A.bruennichi* were extracted with dichloromethane and analyzed by using GC-MS. The analysis of web extracts depicted the existence different chemical molecules that are summarized in Table1. Compounds highlighted in bold existed in web and headspace extracts.

The illustrated Table 1 shows that methyl branched alkanes predominate the extract of virgin females with the domination of the mono methylated compounds ranging from C27 to C35 whereas unbranched alkanes contributed to the next major portion of the web ranging from C16 to C35 indicating the usage of acetate and propionate units as the starting compounds for their biosynthesis. Apart from these two, wax esters comprising of branched acids and unbranched alcohol moieties were identified as third major group of compounds in the web along with trace amounts of trimethyl methylcitrate (**13**) and 3-octanoyloxy-γ-butyrolactone (**14**). Web extracts from the female mated spider also consisted of a similar composition of chemical molecules

except from the slight increase in the percentage of wax esters and dimethylated alkanes were observed. Trimethyl methylcitrate (**13**) remained as virgin specific compound whereas 3-octanoyloxy-γ-butyrolactone (**14**) was also identified in the webs of mated females.

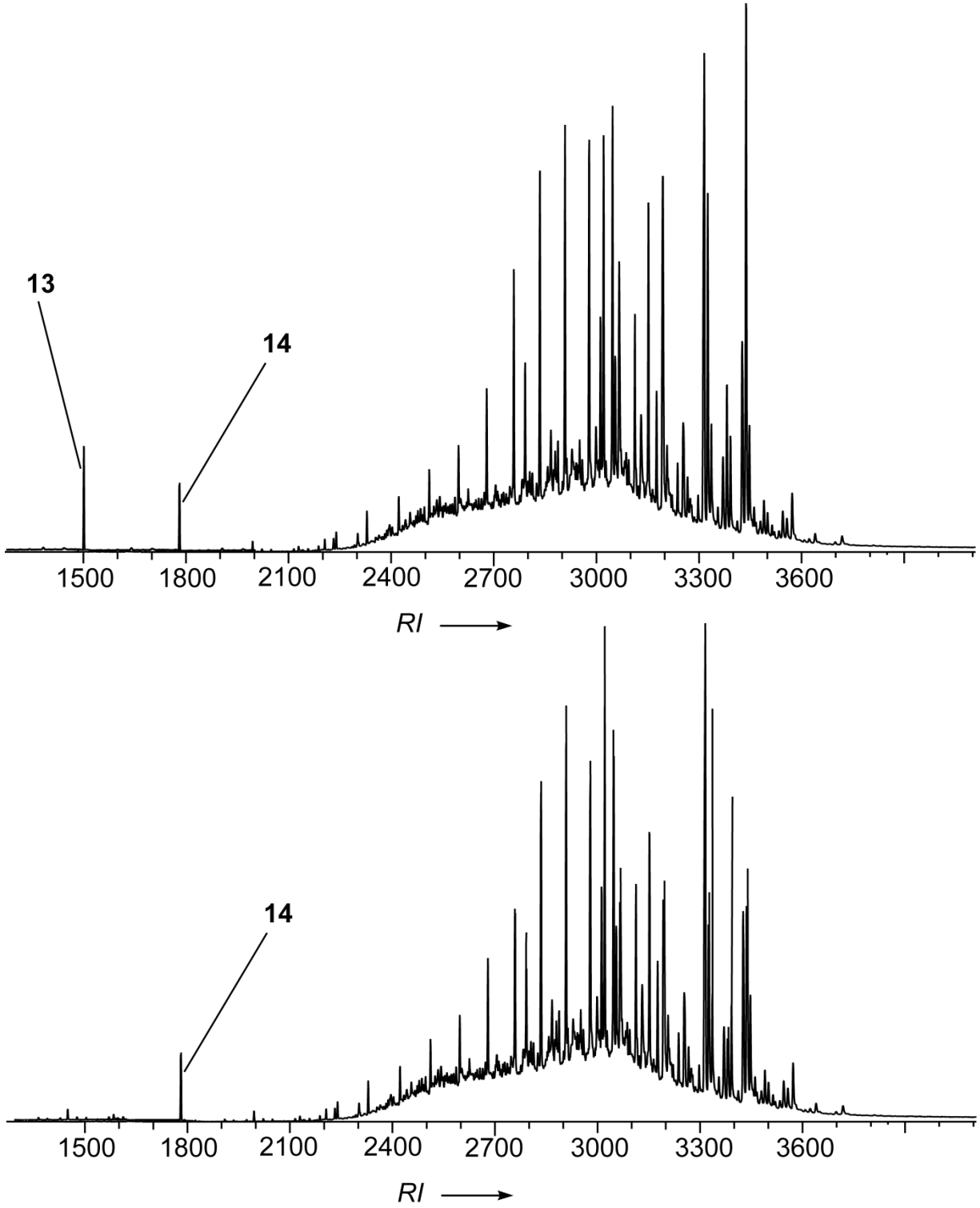


Figure 9. Total ion chromatograms from the web extracts of *Argiope bruennichi* Virgin female (top); Mated female (bottom).

Table 1. Chemical composition of female *Argiope bruennichi* web and headspace extracts (V= Virgin, M = Mated, HS = Headspace extracts, intensities in % to the peak of highest intensity).

RI	Compound	WEB V	WEB M	HS V	HS M
1518	**Trimethyl methylcitrate**	0.6	-	1.6	-
1527	**Trimethyl methylcitrate**	Tr	-	Tr	-
1600	Hexadecane	Tr	-	-	-
1623	**Tetradecanal**	Tr	-	Tr	-
1700	**Heptadecane**	Tr	-	Tr	-
1800	Octadecane	Tr	Tr	-	-
1809	3-octanoyloxy-γ-butyrolactone	0.5	0.4	-	-
1827	**Hexadecanal**	Tr	Tr	Tr	Tr
1900	Nonadecane	0.2	Tr	-	-
2000	**Icosane**	0.4	Tr	0.1	0.2
2100	**Henicosane**	0.6	0.3	0.4	0.2
2200	**Docosane**	1.0	0.3	0.3	0.2
2300	**Tricosane**	1.2	0.8	0.6	0.3
2400	**Tetracosane**	2.1	1.0	0.9	0.7
2500	**Pentacosane**	3.5	2.3	1.7	1.1
2600	**Hexacosane**	4.0	3.2	2.7	3.2
2700	**Heptacosane**	4.1	4.0	2.7	1.9
2762	**2-methylheptacosane**	1.2	1.1	0.8	1.1
2772	3-methylheptacosane	1.6	1.1	-	-
2800	**Octacosane**	5.9	4.9	2.2	1.4
2863	**2-methyloctacosane**	4.0	3.5	1.7	0.9
2872	3-methyloctacosane	-	1.1	-	-
2900	**Nonacosane**	6.2	6.1	3.5	2.9
2916	Heptacosanal	3.0	3.1	-	-
2930	(11/13/15)-methylnonacosane	4.8	3.5	-	-
2947	**5-methylnonacosane**	0.8	-	Tr	Tr
2961	2-methylnonacosane	1.2	1.1	-	-
2972	3-methylnonacosane	1.2	0.8	-	-
3000	**Triacontane**	3.2	2.1	1.7	1.9
3028	**(11/12/13/14/15)-methyltriacontane**	2.6	-	Tr	Tr
3062	**2-methyltriacontane**	2.2	4.6	0.3	0.1
3100	Hentriacontane	2.0	2.4	-	-
3130	(11/13/15)-methylhentriacontane	6.1	4.7	-	-
3150	13,17-dimethylhentriacontane	1.2	0.8	-	-
3155	**9,13-dimethylhentriacontane**	0.8	-	0.1	0.2
3200	Dotriacontane	1.4	1.5	-	-
3228	(12/13/14/15/16)-methyldotriacontane	2.2	3.7	-	-
3249	13,17-dimethyldotriacontane	1.0	1.1	-	-
3261	Tridecyl 2,(4/6/8)-dimethyloctadecanoate	-	1.7	-	-
3263	2-methyldotriacontane	0.6	-	-	-
3300	Tritriacontane	0.8	0.8	-	-
3331	(11/13/15/17)-methyltritriacontane	6.4	7.8	-	-

RI	Compound	WEB V	WEB M	HS V	HS M
3349	(13,17/13,19)-dimethyltritriacontane	3.6	3.2	-	-
3367	Tridecyl 2,(4/6/8)-dimethylnonadecanoate	2.2	6.3	-	-
3400	**Tetratriacontane**	0.6	0.9	Tr	Tr
3427	(13/14/15/16/17)-methyltetratriacontane	1.6	2.3	-	-
3447	**(13,19/15,21)-dimethyltetratriacontane**	1.8	2.0	0.9	1.3
3465	Tetradecyl 2,(4/6/8)-dimethylnonadecanoate	1.6	4.0	-	-
3500	Pentatriacontane	0.4	-	-	-
3527	(11/13/15/17)-methylpentatriacontane	2.8	3.3	-	-
3547	(13,19/13,21)-dimethylpentatriacontane	5.9	4.4	-	-

3.7 Chemical composition of web extracts from *Argiope bruennichi* males

Virgin and mated male web extracts were also extracted with dichloromethane and analyzed using GC-MS showed that the wax esters containing dimethyl branched acids and *n*-alcohols dominated the web of both mated and virgin with the one from latter being slightly more in percentage. Their chain length varyied from C17 to C37 followed by several other classes of compounds that include branched and unbranched alkanes, aldehydes, acids and 1-methoxyalkanes (Table 2). Compounds highlighted in bold existed in web and headspace extracts.

Table 2.Chemical composition of male *Argiope bruennichi* web and headspace extracts (V= Virgin, M = Mated, HS = Headspace extracts, intensities in % to the peak of highest intensity)

RI	Compound	WEB V	WEB M	HS V	HS M
1200	**Dodecane**	Tr	Tr	Tr	Tr
1216	**Decanal**	0.2	0.1	Tr	Tr
1300	**Tridecane**	Tr	Tr	Tr	Tr
1317	**Undecanal**	0.2	0.1	0.1	0.1
1400	Tetradecane	Tr	Tr	-	-
1419	Dodecanal	0.1	Tr	-	-
1500	**Tetradecane**	0.2	0.1	0.2	0.2
1521	Tridecanal	0.2	0.1	-	-
1600	**Hexadecane**	Tr	Tr	Tr	Tr
1623	**Tetradecanal**	0.2	0.1	Tr	Tr

RI	Compound	WEB V	WEB M	HS V	HS M
1700	**Heptadecane**	0.2	0.1	0.3	0.2
1725	**Pentadecanal**	Tr	Tr	Tr	Tr
1800	Octadecane	Tr	Tr	-	-
1827	**Hexadecanal**	1.0	0.6	0.1	0.2
1900	**Nonadecane**	0.1	0.1	0.3	0.1
1930	**Heptadecanal**	0.1	Tr	0.2	0.3
2000	**Icosane**	0.1	Tr	Tr	Tr
2016	Hexadecanoic acid	Tr	-	-	-
2032	Octadecanal	0.9	0.5	-	-
2100	**Nonadecanal**	0.2	0.1	0.2	0.3
2134	Henicosanal	0.1	0.1	-	-
2162	**2-methylhenicosane**	Tr	-	Tr	Tr
2171	3-methylhenicosane	Tr	-	-	-
2200	**Docosane**	0.2	0.2	0.3	0.1
2216	Octadecanoic acid	Tr	-	-	-
2236	Icosanal	0.4	0.3	-	-
2263	**2-methyldocosane**	0.1	0.1	0.1	0.2
2300	**Tricosane**	1.7	1.0	0.4	0.1
2334	**11- methyltricosane**	0.1	0.1	0.6	0.3
2348	5-methyl tricosane	0.1	0.1	-	-
2362	**2-methyltricosane**	0.2	0.1	0.1	0.4
2372	3-methyltricosane	0.1	0.1	-	-
2400	**Tetracosane**	0.4	0.3	0.9	0.3
2416	Icosanoic acid	Tr	0.1	-	-
2433	**1-methoxydocosane**	0.3	0.2	Tr	Tr
2440	Docosanal	0.4	0.3	-	-
2462	**2-methyltetracosane**	1.9	1.0	1.1	1.7
2500	**Pentacosane**	2.2	1.4	2.1	1.4
2540	7-methylpentacosane	0.1	0.1	-	-
2562	**2-methylpentacosane**	0.4	0.4	0.9	0.6
2572	3-methylpentacosane	0.3	0.2	-	-
2600	**Hexacosane**	0.5	0.4	0.5	0.3
2618	Docosanoic acid	Tr	0.1	-	-
2635	**1-methoxytetracosane**	3.7	2.6	Tr	Tr
2663	2-methylhexacosane	6.2	4.5	-	-
2700	**Heptacosane**	2.5	1.8	3.7	2.6
2730	(11/13)-methylheptacosane	1.3	1.1	-	-
2735	**1-methoxypentacosane**	1.3	1.1	Tr	Tr
2738	7-methylheptacosane	1.3	1.1	-	-
2748	5-methylheptacosane	0.1	0.2	-	-
2762	**2-methylheptacosane**	0.5	0.4	0.3	0.6
2772	3-methylheptacosane	0.7	0.6	-	-
2800	**Octacosane**	0.6	0.5	0.4	0.5
2834	**1-methoxyhexacosane**	10.5	7.2	Tr	Tr
2863	**2-methyloctacosane**	7.5	6.0	2.5	3.0
2872	3-methyloctacosane	-	0.2	-	-
2900	**Nonacosane**	1.6	1.3	0.9	1.1

RI	Compound	WEB V	WEB M	HS V	HS M
2929	(11/13/15)-methylnonacosane	0.6	0.6	-	-
2937	(7/9)- methylnonacosane	1.4	1.1	-	-
2948	5- methylnonacosane	0.2	0.2	-	-
2952	(15/11,17)-di methylnonacosane	0.2	0.5	-	-
2962	**9,19-dimethylnonacosane**	0.4	0.6	0.6	0.3
2973	3- methylnonacosane	0.6	0.5	-	-
3000	**Triacontane**	0.3	0.5	0.4	0.2
3004	3,9-dimethylnonacosane	0.2	-	-	-
3028	(11/12/13/14/)-methyltricontane	0.2	0.2	-	-
3035	**1-methoxyoctacosane**	0.9	1.2	Tr	Tr
3059	**2-methyltriacontane**	1.4	1.9	0.8	1.1
3069	Dodecyl 2-methyloctadecanoate	1.2	1.2	-	-
3100	**Hentriacontane**	0.3	0.4	0.2	0.4
3128	(11/13/15)-methylhentriacontane	0.4	0.6	-	-
3138	(7/9)- methylhentriacontane	0.1	0.2	-	-
3151	11,17-dimethylhentriacontane	0.2	0.3	-	-
3169	Tridecyl 2-methyloctadecanoate	11.2	12.2	-	-
3189	Tridecyl icosanoate	0.2	0.5	-	-
3207	tetradecyl nonadecanoate	0.2	0.4	-	-
3265	Tetradecyl 2-methyloctadecanoate	7.7	9.8	-	-
3291	Tetradecyl icosanoate	0.2	0.3	-	-
3312	Tridecyl henicosanoate	-	0.2	-	-
3327	11/13/15-methyltritriacontane	0.2	0.3	-	-
3352	13,19-dimethyltritriacontane	-	0.3	-	-
3369	Tridecyl 2,8-dimethylicosanoate	14.4	18.9	-	-
3397	Tridecyl docosanoate	0.4	0.5	-	-
3415	Tetradecyl henicosanoate	-	0.4	-	-
3491	Tetradecyl 2-methylhenicosanoate	4.4	5.4	-	-
3524	Tetradecyl docosanoate	-	0.2	-	-
3571	Tetradecyl 2-methyldocosanoate	-	0.1	-	-
3640	Tridecyl tetracosanoate	1.5	2.0	-	-
3672	Tridecyl 2-methyltetracosanoate	0.2	0.2	-	-

All the above listed compounds from web of male and female *A.bruennichi* (mated and virgin) were investigated by using GC-MS and structures were confirmed by comparing their mass spectra with those of the standard ones from NIST mass spectral library (version 2.0, 23 July 2008). Supporting conclusions were drawn from the calculation of retention indices. Structure proposals for trimethyl methylcitrate (**13**) and 3-octanoyloxy-γ-butyrolactone (**14**) were done by elucidating the mass

fragmentation pattern, followed by performing derivatization of the latter (discussed in the following sections). Investigations of chemical composition of the volatile extracts obtained by using headspace technique also relied on same methodology.

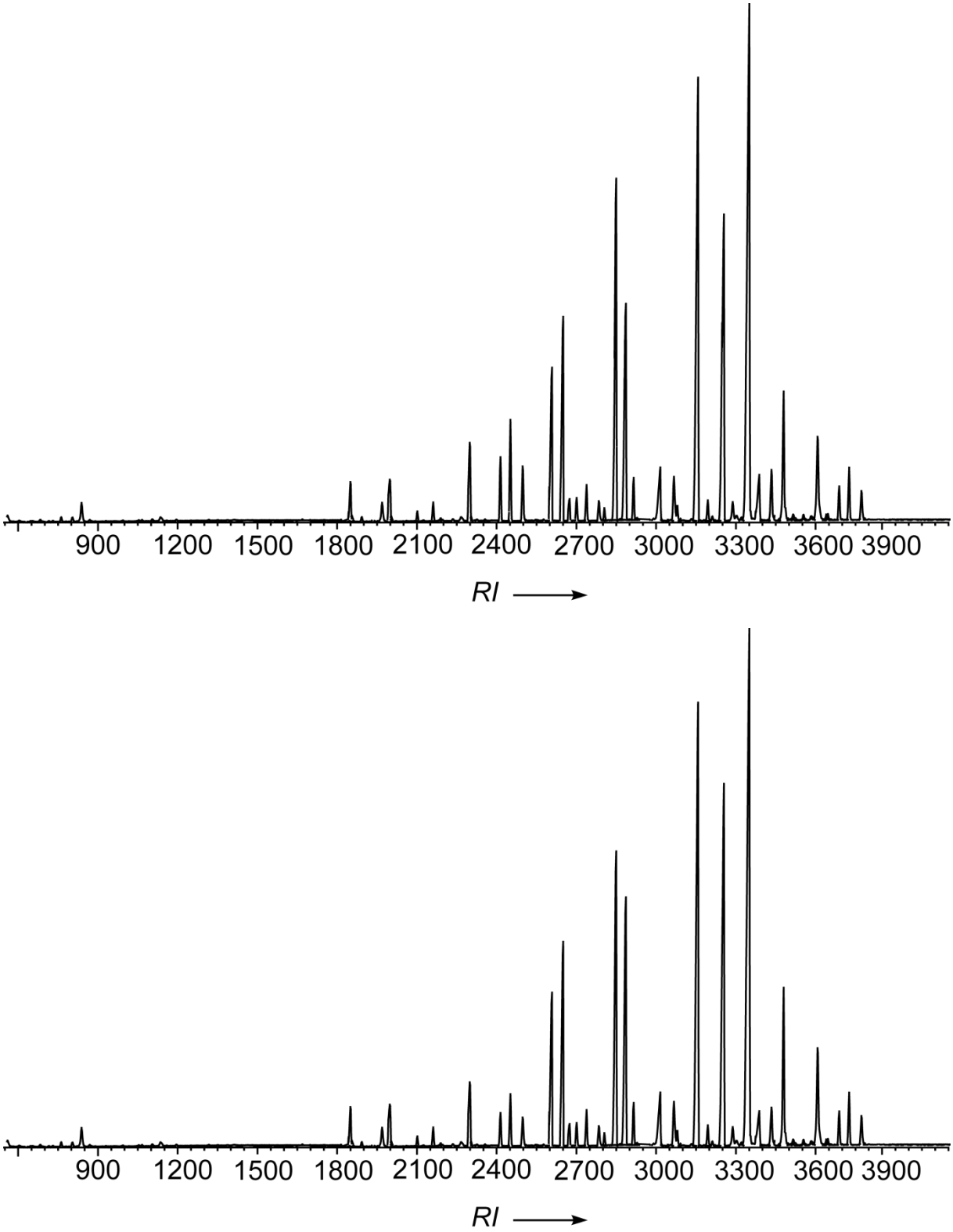


Figure 10. Total ion chromatograms from the web extracts of *Argiope bruennichi* Virgin male (top); Mated male (bottom).

3.8 Extraction of spider volatiles by using headspace technique

For obtaining the head space extracts, male and female *Argiope bruennichi* spiders were confined for 24 hrs in a closed glass chamber as shown in Figure 11. The spiders were provided with a rolled mesh in it to enable their movement within the chamber. The glass chamber is connected via a pipe to an activated charcoal filter that is in turn connected to the pump which pulls the air from the glass chamber so that any volatiles released by the spider are trapped by the charcoal filter. The charcoal filter is then extracted with dichloromethane, and the extracts were analyzed by GC-MS.

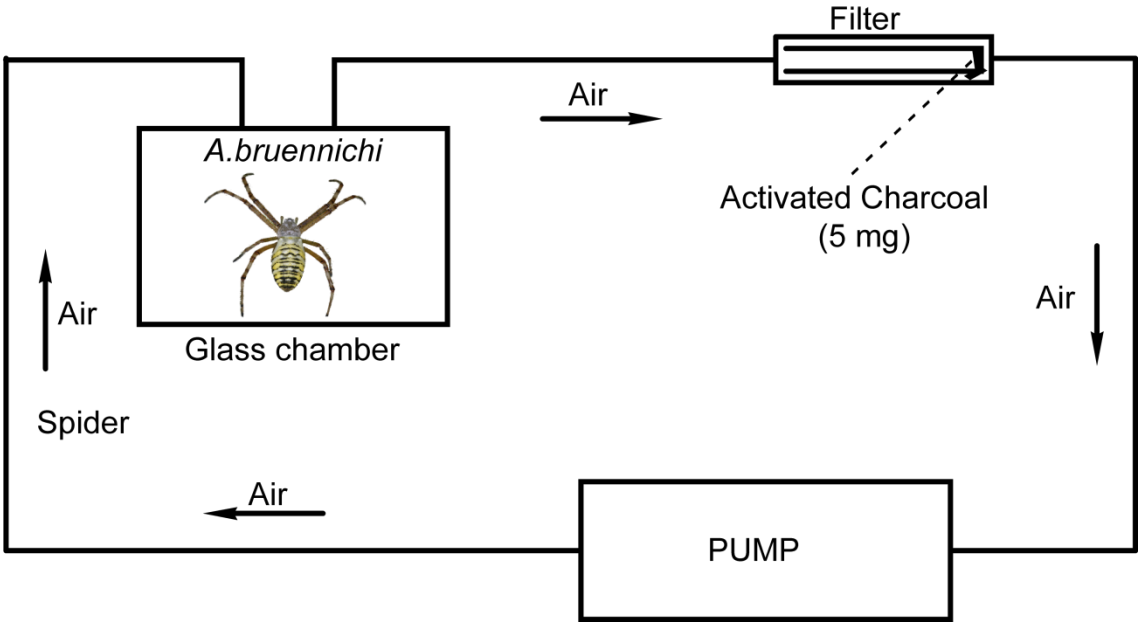


Figure 11. Process of headspace analysis

3.9 Chemical composition of headspace extracts from male and female *Argiope bruennichi* (virgin and mated)

Headspace extracts obtained from male and female *A. bruennichi* (mated and virgin) predominantly consisted of methyl branched and unbranched alkanes (in approximately equal amounts), aldehydes, and trace amounts of methoxy alkanes (table 1& 2 marked in bold). The significant difference in comparison to virgin female web extracts is that the amount of trimethyl methylcitrate (**13**) which was also observed in the virgin female headspace extracts increased drastically. Comparisons involving web and headspace extracts were performed as discussed in the following

section. The compound 3-octanoyloxy-γ-butyrolactone (**14**) was not observed in any of the headspace extracts of *A.bruennichi*.

3.10 Comparison of web extracts from *Argiope bruennichi*

Although several other differences in the hydrocarbon pattern was observed from the web extracts of female and male *Argiope bruennichi*, priority was given to trimethyl methylcitrate (**13**) and 3-octanoyloxy-γ-butyrolactone (**14**) which were exclusively identified only in female web extracts (Figure 10).

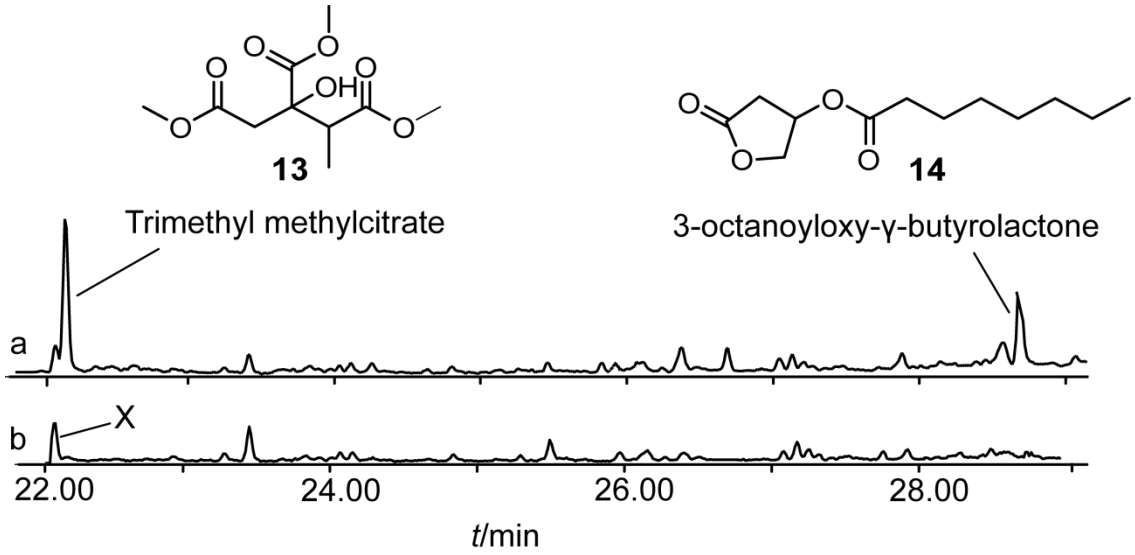


Figure 12. Gas chromatograms from web extracts of *A.bruennichi*
a) Virgin female; b) Sub adult female. X: Impurity

The two compounds **13** and **14** were possible candidates for showing female specific pheromonal activity. None of these two compounds were observed in the web extracts of male virgin or male mated *A.bruennichi*. Trimethyl methylcitrate (**13**) occurred only on virgin webs, along with a much smaller peak at the tail end of this peak showing an identical mass fragmentation pattern indicating the existence of a diastereomer (approximate in the ratio 25:1). Compound **14** was less likely to be a pheromone candidate because it occurred in webs of virgin and mated females.

3.11 Comparison of headspace extracts from *Argiope bruennichi*

Headspace sampling performed with different physiological states of spider *Argiope bruennichi* showed that only virgin females emitted trimethyl methylcitrate (**13**) (isomeric form) whereas 3-octanoyloxy-γ-butyrolactone (**14**) was absent.

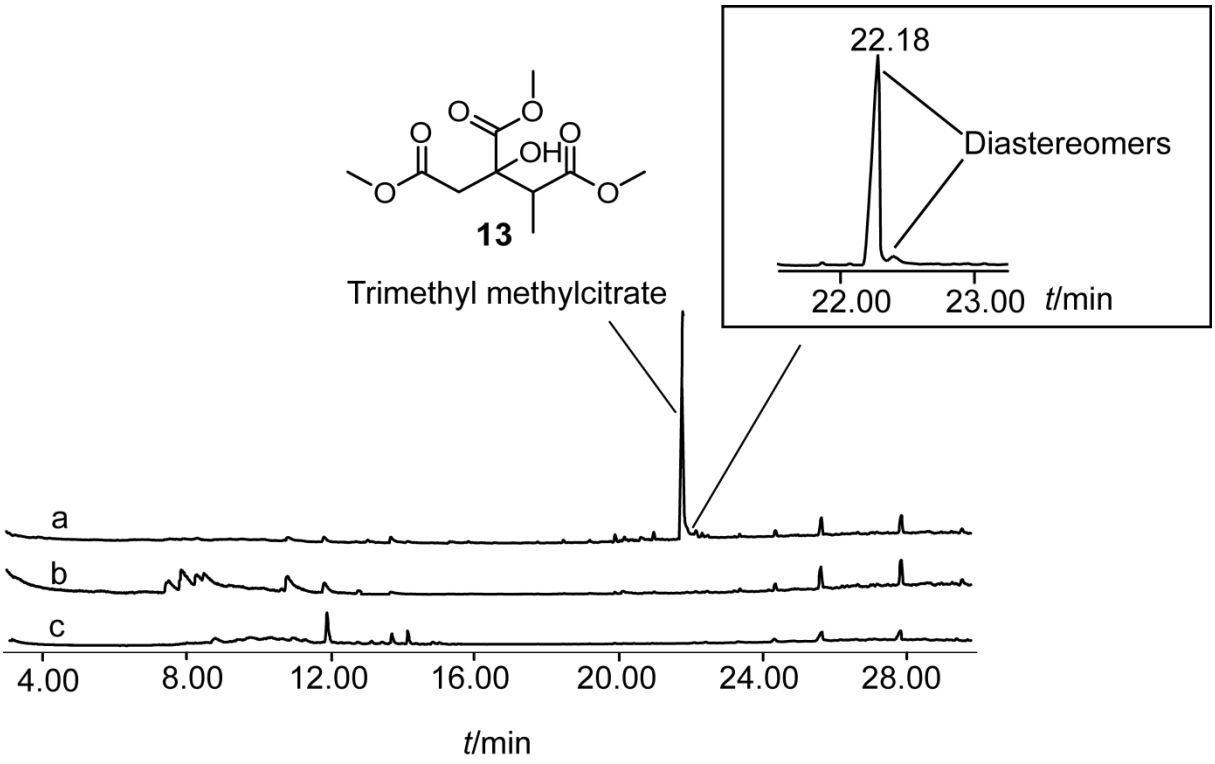


Figure 13. Gas chromatograms from headspace analysis of *Argiope bruennichi*
a) Virgin; b) Mated; c) Sub-adult females

The presence of this compound **13** in the headspace extracts of virgin females led us to develop a hypothesis saying this compound might be a female specific pheromone with activity over long range since it was volatile. The foremost conclusion of this experiment was the considerable increase in the concentration of this compound **13** when compared to its amount in the web extracts which also supported our conception. Trace amounts of these virgin female specific compounds were also identified in the body extracts.[63]

3.12 Structure elucidation of female specific compounds

For the structure confirmation of trimethyl methylcitrate (**13**) and 3-octanoyloxy-γ-butyrolactone (**14**) which were found exclusively in female spiders, mass spectral fragmentation pattern, comparison with closely related molecules, and derivatization with MSTFA proved helpful.

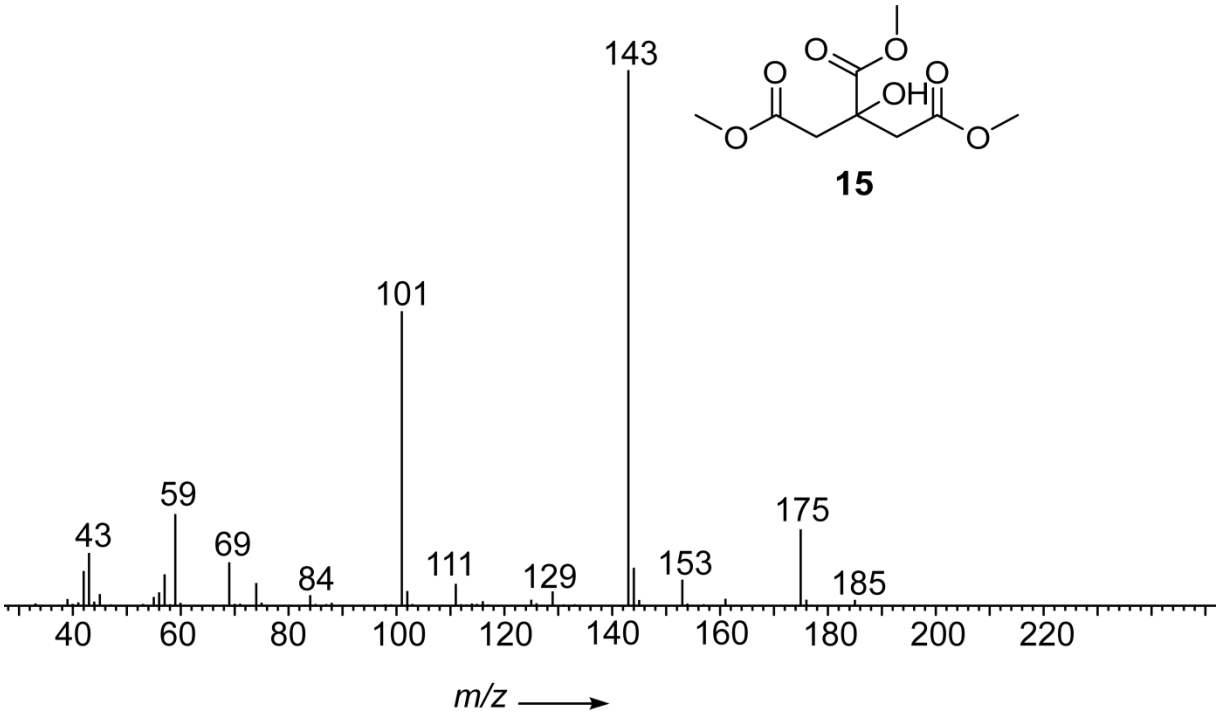


Figure 14. Mass spectrum of trimethyl citrate (**15**)

The mass spectrum of the trimethyl methylcitrate (**13**) (Figure 16) looked similar when compared to that of trimethyl citrate (**15**) (Figure 14) showing an increase in 14 mass units at each major fragment ion indicating the presence of an additional CH_2 group at an unknown position in trimethyl citrate. Considering the observation of an isomer with identical mass spectral data in the natural extract and the achirality of the column in the GC-MS instrument the occurrence of diastereomers with at least two chiral carbon atoms were anticipated. Justified from the above postulation mixed ethyl methyl esters **16** were excluded along with the methylation at the hydroxyl group **17** leaving out the last two possibilities, one being methylation of one of the methylene groups to form trimethyl methyl citrate (**13**), the other being extension of the carbon chain **18** that seemed biosynthetically unlikely.

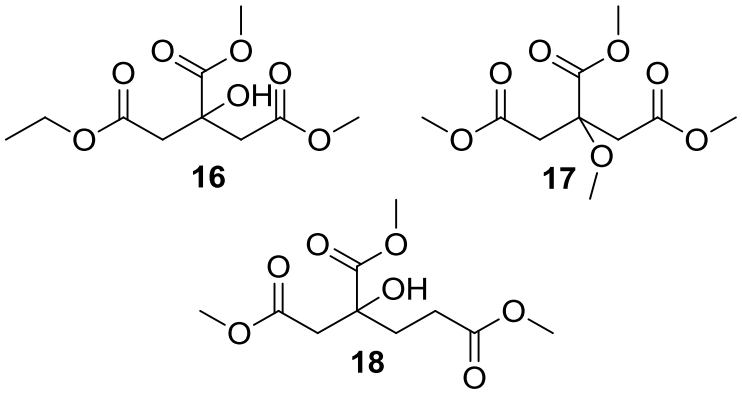


Figure 15. Ruled out possible structures for one of the virgin female specific compound.

The mass spectrum of one of the virgin female specific compound (Figure 16) which was identified to be trimethyl methylcitrate (**13**) can be explained, although the molecular ion (supposed to be 248) was not visible. The ion *m/z* 189 is formed by a α-cleavage of the methoxycarbonyl unit at the quaternary carbon atom. This is followed by an elimination of methanol providing the base ion *m/z* =157. Furthermore, elimination of methyl acetate (74 amu) and methyl propionate (88 amu) from the ion *m/z* = 189 by a McLafferty type rearrangement furnished the ions *m/z* = 115 and 101, respectively.

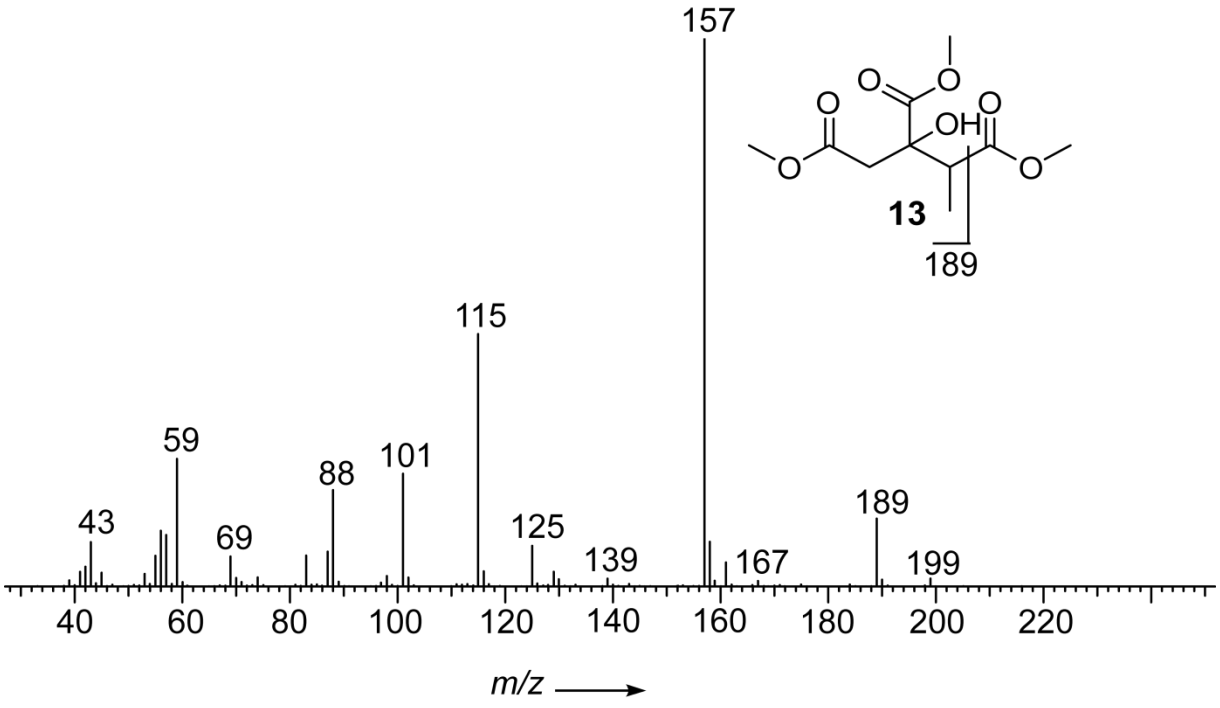


Figure 16. Mass spectrum of trimethyl methylcitrate (**13**)

The molecular structure of trimethyl methylcitrate (**13**) was also confirmed by derivatization with MSTFA which converted the molecule to a TMS-derivative **19** shown below (Figure 17). The molecular ion, formed by inclusion of one TMS-group lead to a shift *m/z* = 248 to *m/z*= 320, can not be observed. By the loss of a methyl group (M^+-15 which is a characteristic cleavage in MSTFA derivatives), result in the ion *m/z* = 305. This spectrum also provided evidence for the existence of one labile hydrogen atom in the molecule indicating the molecular ion of the parent compound to be *m/z* = 248.

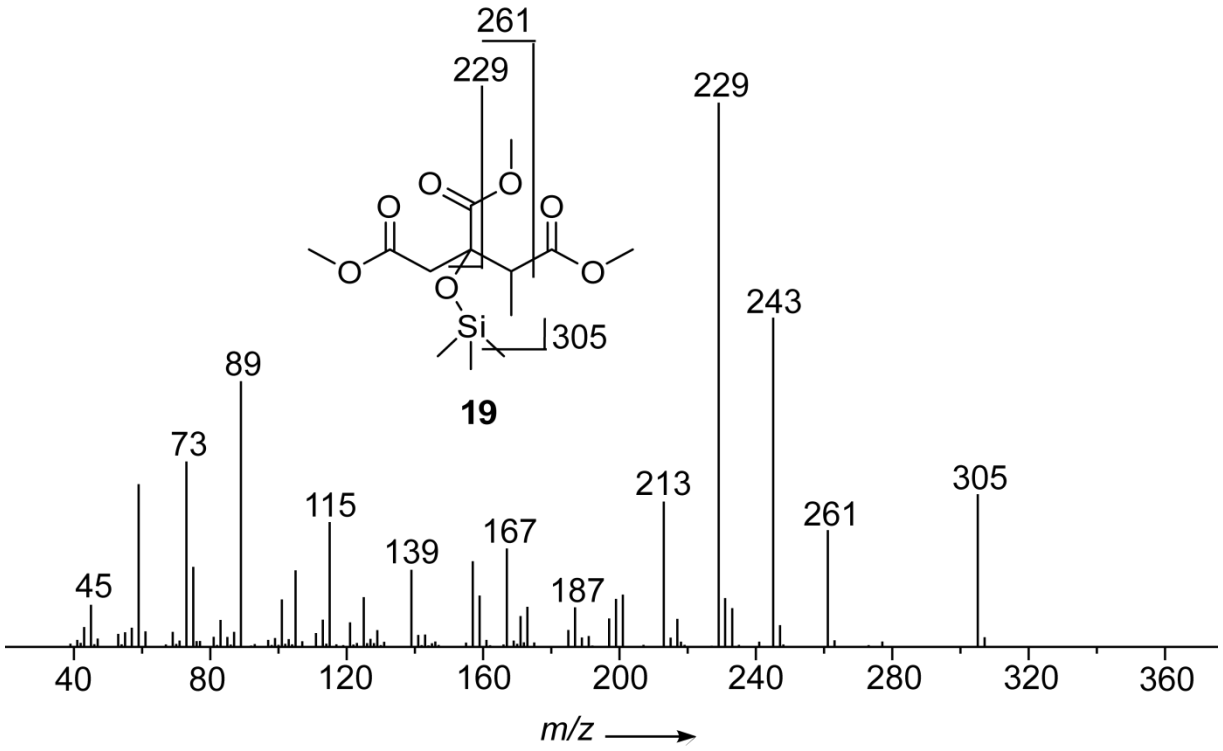


Figure 17. Mass spectrum of the corresponding trimethylsilyl ether of trimethyl methylcitrate **19**

Loss of the methoxy carbonyl group from the quaternary carbon atom results in ion *m/z* = 261. Cleavage of the bond between oxygen and quaternary carbon leads in loss of trimethylsilanol (TMSO) resulting in the base ion *m/z* = 229.

Another female specific compound **14** which was observed only in the silk extracts of virgin and mated females showed the following mass spectrum (Figure 18) with a molecular ion at *m/z* = 228. Its mass spectrum was also characterized by the presence of a major fragment ion *m/z* = 85 (base peak) which is a characteristic peak of the butyrolactone ring.

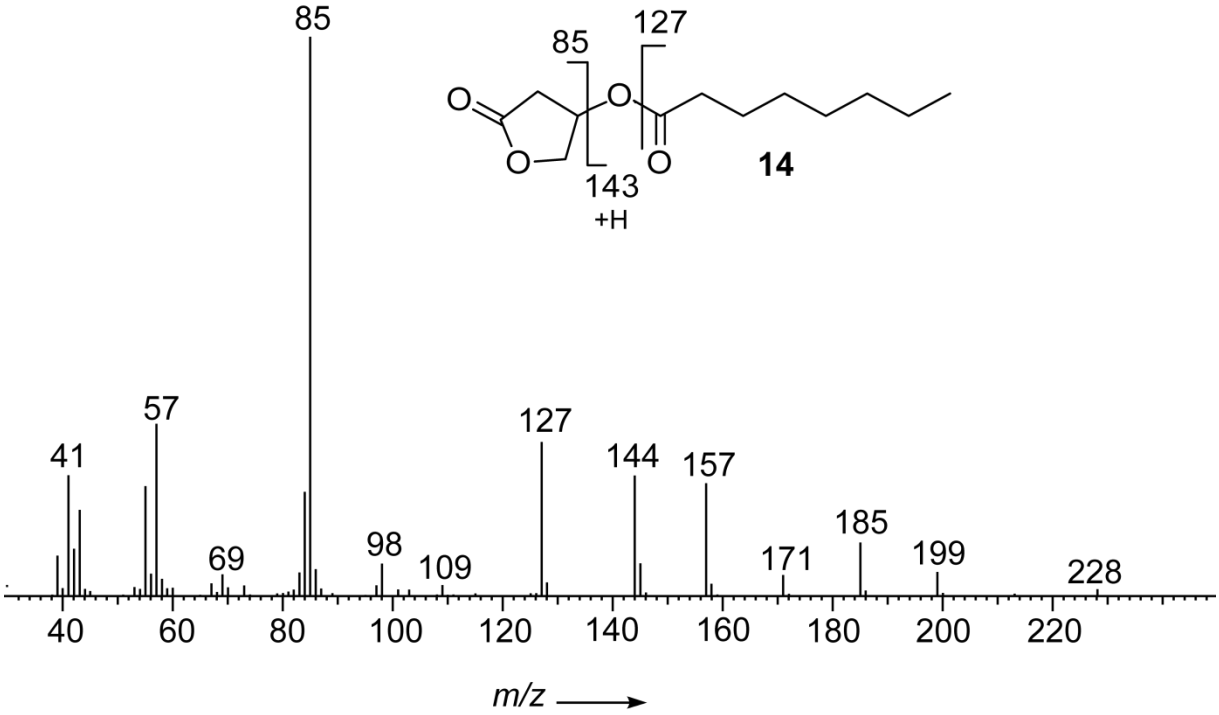


Figure 18. Mass spectrum of 3-octanoyloxy-γ-butyrolactone (**14**)

The mass difference between the base ion and the molecular ion is 144 which refers to the existence of an octanoyl group. This phenomenon can be explained theoretically by the McLafferty rearrangement occurring under elimination of hexene providing the ion *m/z* = 144, analogously to other fatty acid derivatives. The structure is also supported by the existence of mass fragment ion *m/z* = 127 (α-cleavage furnishing octanoyl moiety). From the mass spectrum and the above considerations made, the structure of the second female specific compound was proved as 3-octanoyloxy-γ-butyrolactone (**14**).

Among the two identified female specific compounds from *Argiope bruennichi* trimethyl methylcitrate (**13**) is a structural analog to citric acid and hence closely related to primary metabolites, while 3-octanoyloxy-γ-butyrolactone (**14**) is previously unknown natural compound.

3.13 Synthesis of trimethyl methylcitrate

Trimethyl methylcitrate (**13**), one of the female specific compounds existing in diastereomeric form, is characterized by retention index of 1518 (major isomer) and 1527 (minor isomer). Although from mass spectral interpretations and derivatization a structural proposal was made, synthesis aiming at this molecule has to be performed in order to establish the proposed structure, to provide enough material for performing extensive bioactivity studies, and to assign the absolute configuration of the natural compound.

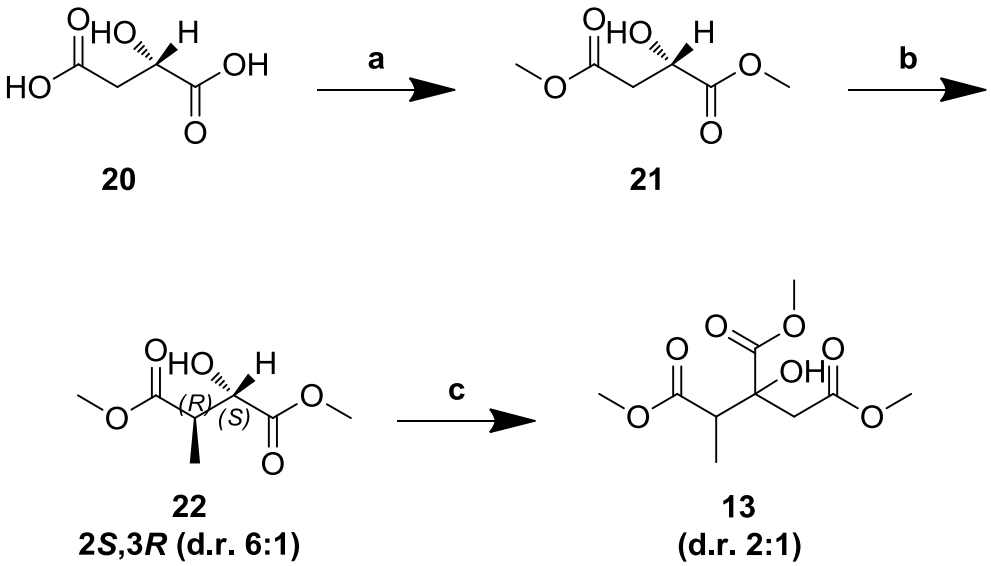
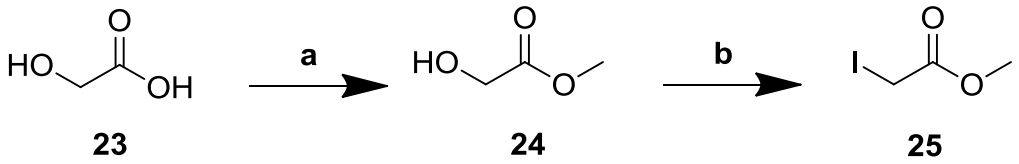


Figure 19. Synthesis of racemic trimethyl methylcitrate (**13**). **a**) MeOH, HCl, 80°C, 88% **b**) LDA, methyl iodide, -78°C, 80% **c**) Li-HMDS, -78°C, methyl 2-iodoacetate, 12%.

Chiral building block (*S*)-malic acid (**20**) was used as starting material which was esterified on both acid functionalities to yield (*S*)-dimethyl malate (**21**) in high percentage without loosing any of the stereogenic information.[64] Thus, obtained (*S*)-dimethyl malate (**21**) was deprotonated at α-position (CH_2 position) with a strong base at deep temperatures, generating an enolate which was captured ultimately by methyl cation (derived from methyl iodide) resulting in the formation of a new chiral center. This molecule **22** with two chiral centers existed in diastereomeric form in the ratio of 6:1, where the 2*S*,3*R* diastereomer predominated in the mixture (97% of monomethylated product).[64] Although the yield of this reaction was near to satisfactory, using higher amounts (2.5 equivalents) of base provided the

dimethylated products, while using lesser amounts (1 equivalent) ended up in remains of the educt **21** in considerable amounts. Conversion of dimethyl (2*S*,3*R*)-3-methylmalate (**22**) to the trimethyl methylcitrate (**13**) was performed by using LDA which enabled the deprotonation at the α-carbon atom of the C-2 chiral center. The enolate then substitutes the iodide of methyl-2-iodoacetate (**25**) in a S_N2 reaction.



23 **24** **25**

Figure 20.Synthesis of methyl 2-iodoacetate (**25**) **a**) abs. MeOH, H_2SO_4, 80°C, 74% **b**) PPh_3, imidazole, I_2, THF, 0°C, 65%.

The synthesis of methyl 2-iodoacetate (**25**) proceeded starting with commercially available glycolic acid (**23**) which was transformed into its corresponding ester methyl 2-hydroxyacetate (**24**) by acid catalyzed esterification[65] in good yield, followed by the conversion of the hydroxy functional group into the respective iodide by reaction with iodine and imidazole[66-69] giving the desired compound **25**. The enolate ion generated from dimethyl (2*S*,3*R*)-3-methylmalate (**22**) was then coupled with **25** leading to the formation of the female specific trimethyl methylcitrate (**13**).

The stereoinformation at the parental chiral carbon C-3 in **22** was lost during this process because of the enolate generation resulting in the formation of the 2:1 diastereomeric product ratio of trimethyl methylcitrate (**13**). Alkylation in strongly basic conditions also epimerized the C-3 stereogenic center.

Although the above discussed route delivered a racemic product it proved the structural prediction and was able to establish the exact structure for the female specific compound trimethyl methylcitrate (**13**). When compared with naturally existing **13** the synthetic sample showed identical mass spectral fragmentation pattern and identical retention indices. Nevertheless, further attempts were made to elucidate the absolute configuration of the compound **13**.

The role played by chirality in pheromone science is significant. Extensive reviews over the decades have shown that many pheromone compounds show stereochemical elements and additional features like double bonds or chiral carbon

centers would further enrich their diversity.[70] Since the female specific compound trimethyl methylcitrate (**13**) contains two chiral carbon atoms four stereoisomers exist.

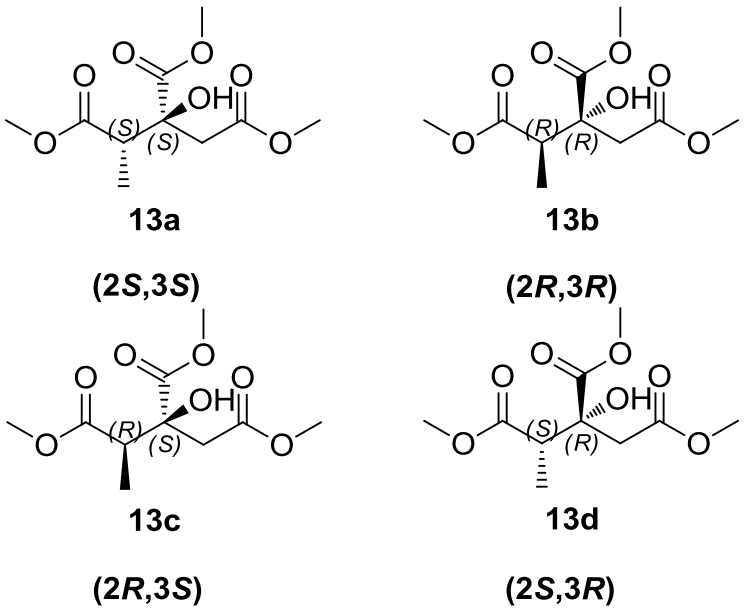


Figure 21. The four possible stereoisomers of trimethyl methylcitrate (**13**).

The next aim was to develop a more stereospecific route to enrich the isomeric purity of compound **13** and also to assign its absolute. Because, compound **13** in the natural extracts was obtained only in minute quantities (nanogram scale) and also as mixture in combination with several other compounds (Table 1 and 2), purification of that compound was beyond the scope and stereochemical assignment became more complicated. Moreover the naturally occurring compound was present in two diastereomers as shown by a GC on a non chiral phase. Which of the four possible isomers occur in the natural extract can only be proven by chiral resolution studies. The best possibility to carry out such a determination was a synthesis starting from a compound of known absolute configuration, followed by comparisons of the gas chromatographic behavior of the synthetic and natural material on an optically active stationary phase. However synthesis, and assigning the configuration of the natural compound is itself a major task along with establishing the relationship between the absolute configuration and bioactivity.[71,72]

The synthesis of the enantiomers was planned around Seebach'schiral relay method starting from (*S*)-malic acid (**20**).[76,81] Thus acid catalyzed acetalization of pivalaldehyde with enantiopure (*S*)-malic acid (**20**) furnished *cis*-acetal **26**. Since the proton on the α-carbon atom is *trans* to the *tert*-butyl group in **26** it was anticipated

that deprotonation will occur easily to form a chiral enolate, which can then be alkylated with the retention of configuration giving **27**. This enolate was planned to react with 1-bromo-2-butene in a S_N2 reaction. In this reaction the enolate should preferentially attack on the γ-position of the bromide. Then the alkene should be oxidized and transformed into ester group.

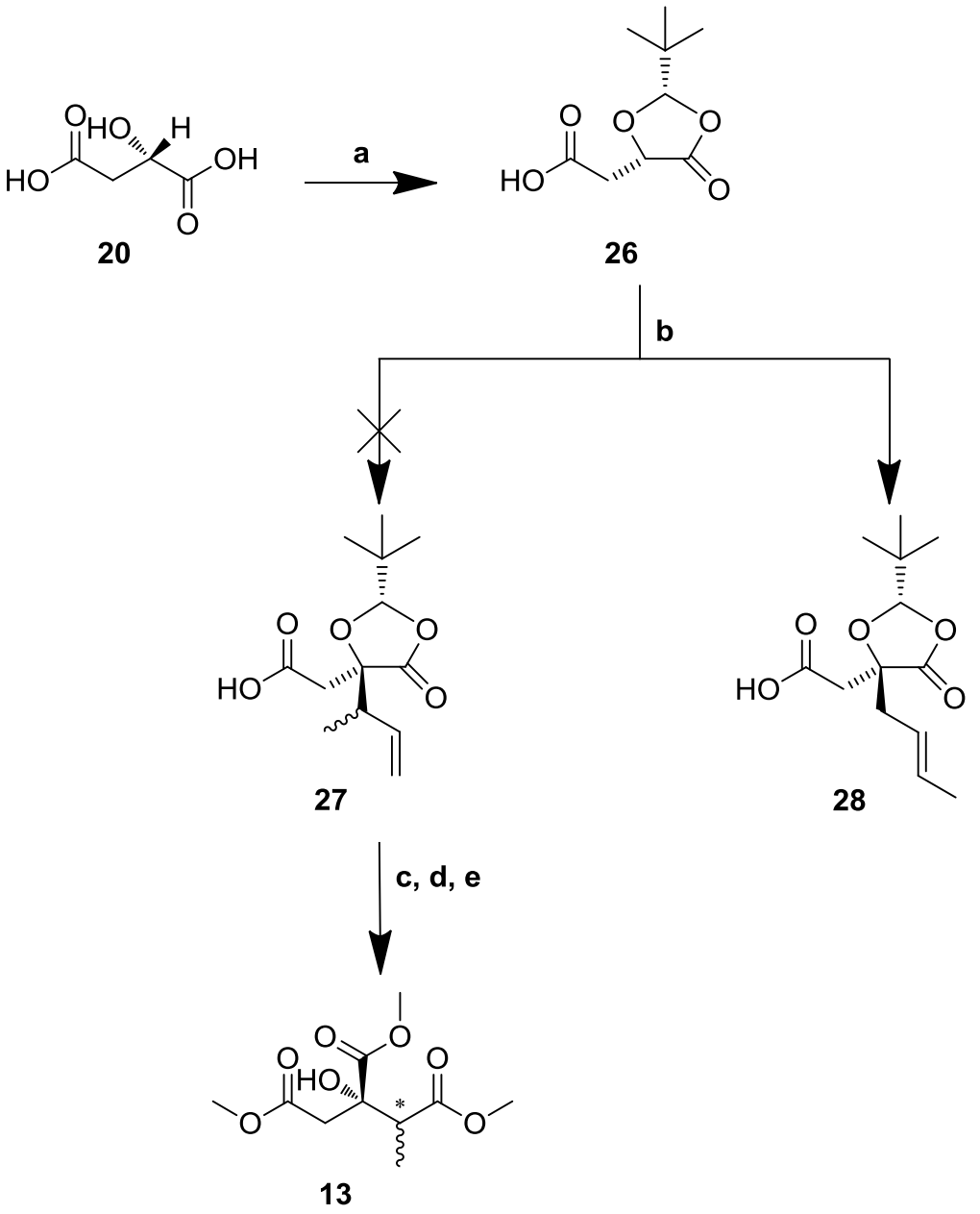


Figure 22. Unsuccessful approach to synthesize trimethyl methylcitrate **13** **a**) H_2SO_4, pivalaldehyde 61% **b**) Li-HMDS, -78°C, 1-bromo-2-butene **c**) $BF_3{\cdot}Et_2O$, MeOH **d**) $RuCl_3$, $NaIO_4$, CCl_4, MeCN, H_2O **e**) EDC, MeOH

However, the results showed that the deprotonation occurred as expected but instead of the γ-attack, α-attack was observed eventually ending up with compound **28** which was not desired (Figure 22).

Therefore, the initial route was extended using the Seebach methodology to establish the structure of compound **13**. This strategy changed the order of events. Now first introducing the methyl group at C-2 in (*S*)-malic acid (**20**) and then using allyl bromide as alkylating agent, thus avoiding problems with α/γ-selectivity (Figure 22).

Again (*S*)-malic acid (**20**) was converted into its corresponding methyl ester **21.** This compound was methylated[73] at the α-position by using LDA as base to give methylated dimethyl malate **22** in a diastereomeric ratio of 6:1 in favor of the 2*S*,3*R* isomer predominating in the mixture.[64] Hydrolysis[74] of **22** gave the respective acid **29** which was then treated with pivalaldehyde to convert[75] it into the thermodynamically more stable *cis* dioxolanone **30** that was subsequently alkylated with allyl bromide yielding **31** according to the chiral relay method developed by Seebach et. al.[76] Treatment with boron trifluoride diethyl etherate in methanol cleaved the acetal and simultaneously esterified the acid group in a single step to give **32**. Oxidation of the double bond in **32**[77,78] with ruthenium tetroxide furnished the acid **33**, but in poor yield only. Esterification[79] with methanol yielded trimethyl (2*S*,3*R*)-methylcitrate **13c** as the major enantiomer (6:1 diastereomeric ratio In favor of 2*S*,3*R* proven by GC-MS and ^{1}H,^{13}C-NMR)(Figure 24).

Since Seebachs relay chiral method[80,81] stores the stereogenic information at the *tert*-butyl carbon atom in the molecules **30** and **31**, we can state that although the original chiral center was destroyed, it was regained at the end of the synthesis with the same stereo information. The configuration of the final trimethyl methylcitrate (**13**) was assigned to be 2*R*,3*S* and the minor stereoisomer present contained 2*S*,3*S* configuration.

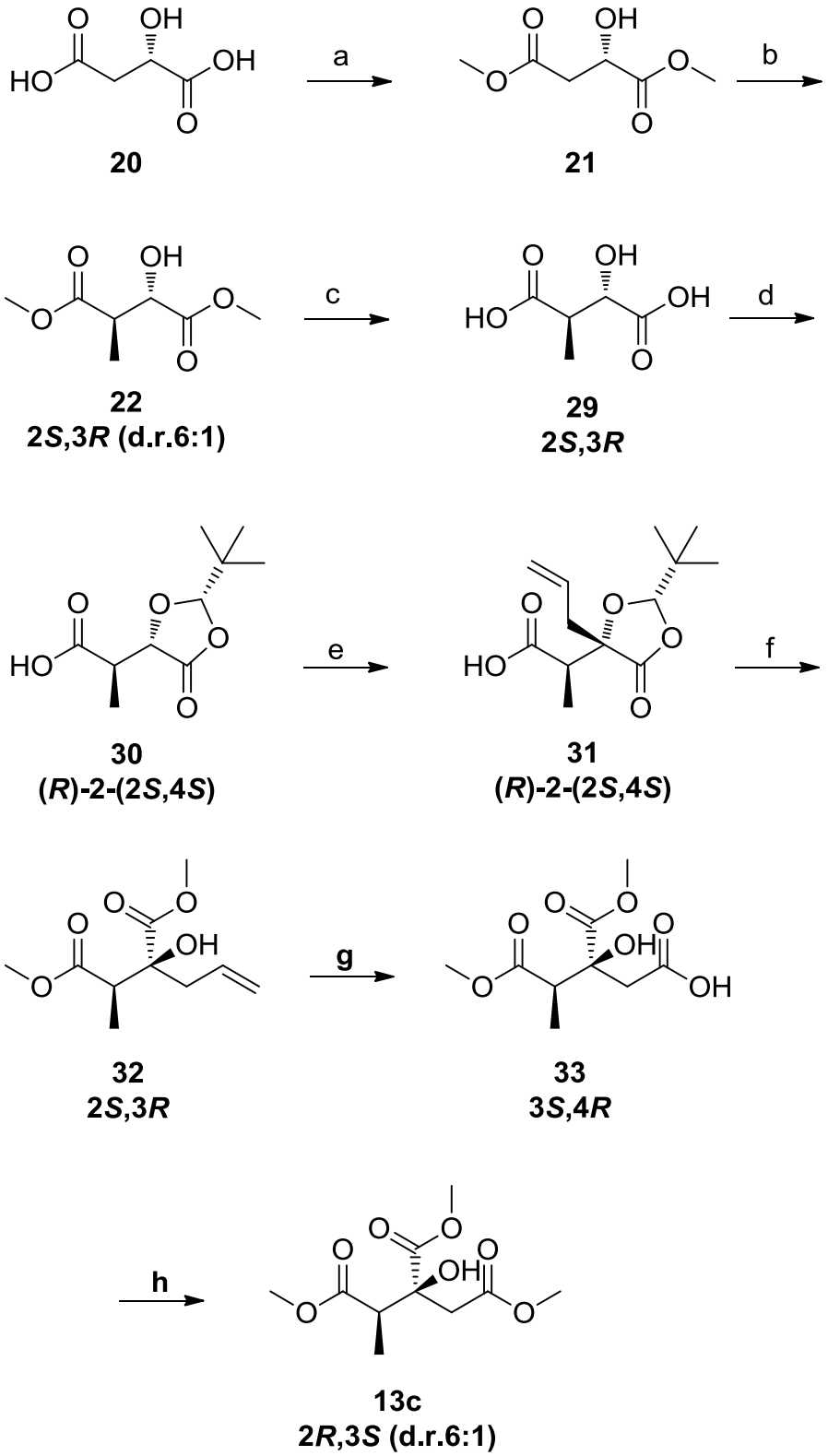


Figure 23. Synthesis of trimethyl (2*R*,3*S*)-methylcitrate (**13c**) **a**) HCl, MeOH, 80°C, 88% **b**) LDA, MeI, -78°C, 80% **c**) 2N KOH, THF:MeOH (1:1), 92% **d**) H_2SO_4, pivalaldehyde, 50% **e**) Li-HMDS, -78°C, allyl bromide, 79% **f**)$BF_3{\cdot}Et_2O$, MeOH, 59% **g**) $RuCl_3$, $NaIO_4$, CCl_4, MeCN, H_2O, 18% **h**) EDC, MeOH, 57%.

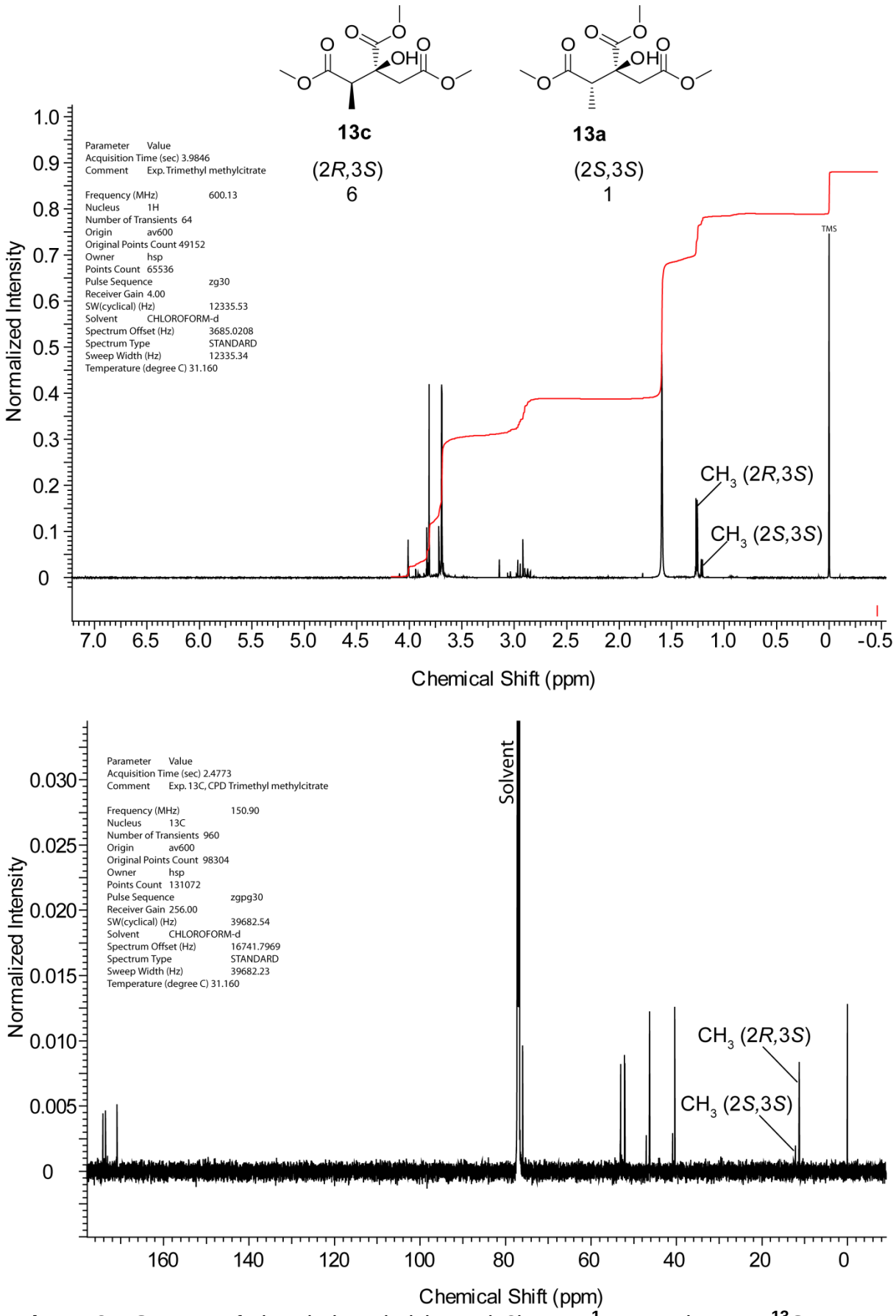


Figure 24. Spectra of trimethyl methylcitrate (**13**): Top: **^{1}H-NMR** bottom: **^{13}C- NMR**

3.14 Chiral resolution of trimethyl methylcitrate

Chiral gas chromatography was then performed to clarify and confirm the absolute configuration of the natural product. The separation of the four enantiomers of trimethyl methylcitrate (**13**) initially proved complicated. Derivatization of the synthetic sample with MSTFA and then implementing chiral resolution accomplished partial separation of corresponding trimethylsilyl ethers of the four enantiomers (Figure 25) on a chiral Lipodex G column. In order to achieve base line separation of the derivatised enantiomers, different conditions were tried which yielded no better seperation.

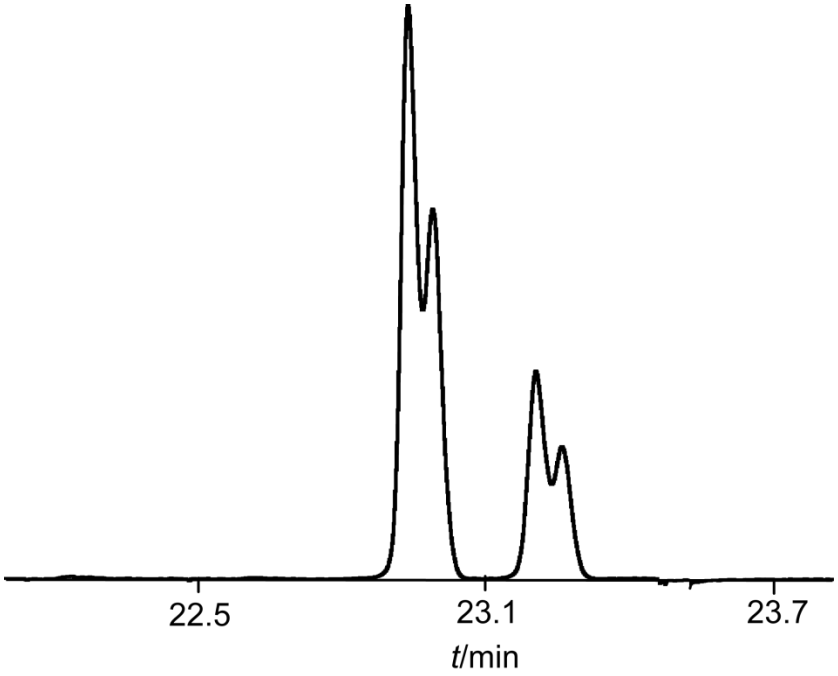


Figure 25. Gas chromatographic separation of the corresponding trimethylsilyl ethers of trimethyl methylcitrate (**13**) on a chiral Lipodex G phase. Temperature program: 140°C for 5 min, then with 1°C/min up to 200°C.

Resolution of synthetic sample **13c** on a chiral Hydrodex-6-TBDMS column (Figure 26) with a very slow program achieved partial separation of the four enantiomers which was sufficient to compare them with the natural extract. The analysis of natural extracts showed that two (2*R*,3*S*)- and (2*S*,3*S*)-enantiomers occurred naturally in a ratio between 6:1 to 25:1. Co-injection of natural extract with synthetic sample **13c** proved the major isomer from the natural extract to be identical to the major isomer in the synthesized compound, trimethyl (2*R*,3*S*)-methylcitrate (**13c**), whilst the minor isomer is the minor synthetic product, trimethyl (2*S*,3*S*)-methylcitrate. The

assignment can be made because the stereogenic center at C-3 was introduced by the starting material **20**.

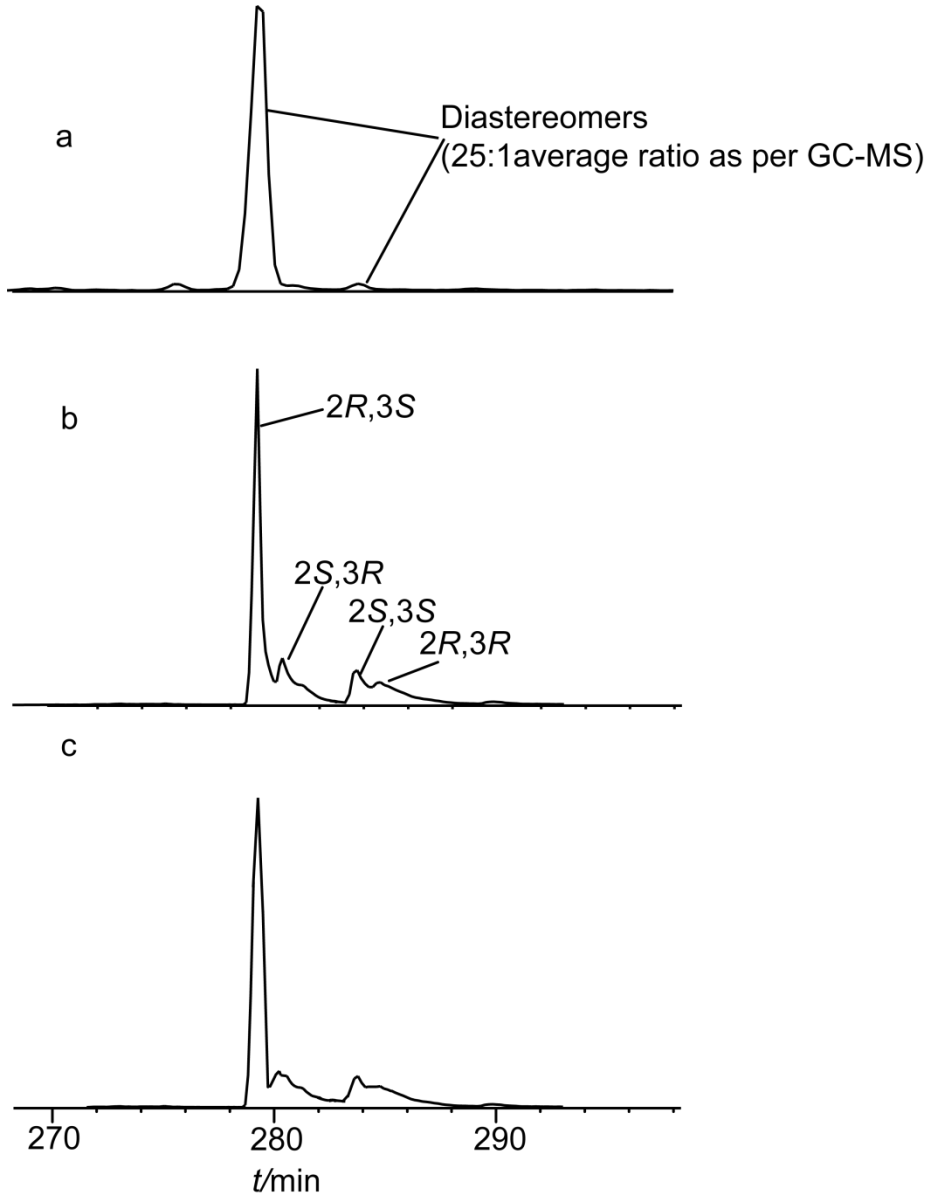


Figure 26. Gas chromatographic separation of trimethyl methylcitrate (**13**) on a chiral hydrodex-6-TBDMS phase. Temperature program: 50°C for 5 min, then with 0.2°C/min to 200°C. **a**) Natural extract; **b**) synthetic **13**; **c**) co-injection.

3.15 Bioactivity of trimethyl methylcitrate

The presence of trimethyl methylcitrate (**13**) in the body extracts, web extracts and headspace extracts of virgin female *Argiope bruennichi* led to the hypothesis that this compound might be a female pheromone. To prove this interpretation, a two chamber choice test was performed by PD Dr. G. Uhl from the University of Bonn.

Female virgin webs were used in this experiment to evaluate the behavior responses from male spiders (virgin & mated). Several investigations conducted in this manner concluded the existence of a volatile pheromone molecule on the webs of virgin females. Observations from these trials also proved that female virgin webs can induce courtship display in males.

Experiments with compound **13** in a Y-tube olfactometer failed, because the spider did not behave properly in this setup. Therefore a trapping bioasaay of male spiders in field was designed which was never attempted earlier in case of spiders.

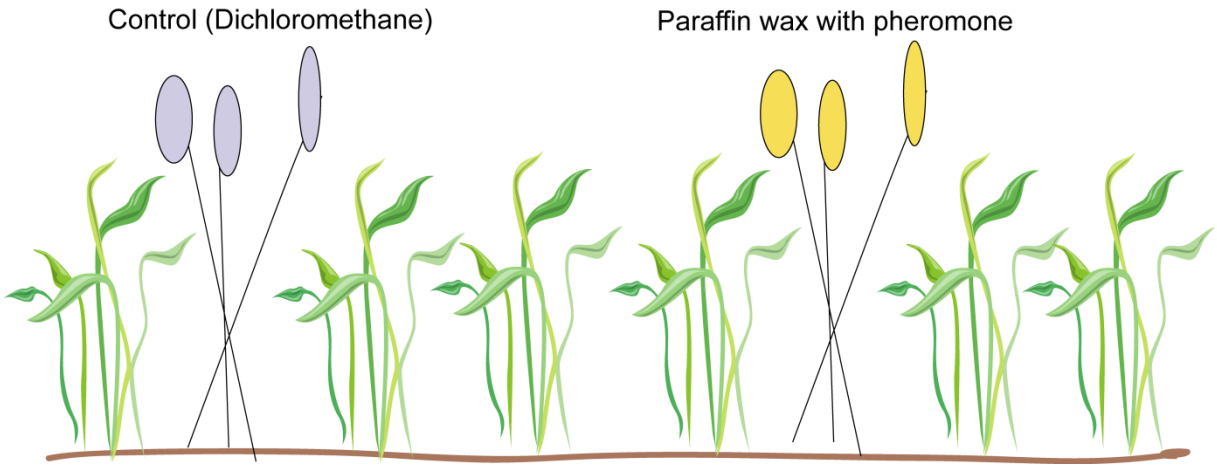


Figure 27. Sketch illustrating the appearance of tripod traps in the field

Tripod traps made out of three barbecue sticks were loosely bound and crossed around two thirds of their length, with their ends orientated in three different directions to resemble grass like plants(Figure 27 & 28). Each of the stick is coated at the top end with paraffin in order to prevent the fast evaporation of the synthetic material. These traps were placed in a grassy open field near Bonn, Germany, ($50^0$45'7.63"N; area 0.25 km^2) where *Argiope bruennichi* occurs in large numbers during the warmer periods of July and August. Different concentrations of synthetic 6:1 mixture of (2*R*,3*S*) and (2*S*,3*S*) of **13c** diluted in dichloromethane were applied equally on the

three sticks of each trap. A second trap was always used as control which contained only dichloromethane placed at a distance of 40-50 cm. Each trap phase or a single test phase was conducted for 30 minute duration during which the approach and behavior of the male spiders was recorded. After each trial the males attracted were caught and released at the end of the day so that they did not interfere with the traps being performed later in the day. After each trail the traps were moved 5-10 m to execute another trap experiment.

Figure 28. Tripod trap placed in a meadow. (Photo: Satya Chinta)

A minimum of four and a maximum of ten traps were used for each concentration in order to arrive at a conclusion. Conclusively 56 traps (Table3) were placed all together of which 35 proved significantly successful in attracting 75 males. Male spiders were seen climbing, approaching and attracted towards the traps containing different concentrations of a synthetic 6:1 mixture of (2*R*,3*S*) and (2*S*,3*S*) **13**. Approaching males climbed the wooden sticks, which was recorded as successful attraction. The variability shown in the trap experiments can result from varying concentrations of the test compounds and also the availability of males in the vicinity of the trap. Moreover, 96 % of males showed behavioral bouts that also occur during courtship (application of silk strands from the paraffin ends to the vegetation, jerking and abdomen vibrations). The single male that was attracted during the seven test trials at the lowest concentration of 0.15 µg also showed courtship behavior. In none

of the test trails male spiders were seen approaching or climbing the control trap which proved that trimethyl methylcitrate (**13**) is the female pheromone of *Argiope bruennichi* spider.

Table 3: Results of bioassays performed in the field.

Amount [µg/100µl]	Trials	Successful trials (%)	Attracted males
25.00	10	10 (100)	34
12.50	10	8 (80)	19
2.25	5	3 (60)	7
1.25	10	5 (50)	6
0.625	4	4 (100)	5
0.30	10	4 (40)	4
0.15	7	1 (14)	1

The attractivity of the pheromone was concentration dependent. Attraction of male spiders towards the traps containing test sample of less than 1 µg was low, but still

Figure 28. Males of *Argiope bruennichi* attracted on to the trap containing synthetic 6:1 mixture of (2*R*,3*S*) and (2*S*,3*S*) trimethyl methylcitrate (**13**). (Photo: Julia Lux)

observable. Higher concentrations attracted more males which was also proven by a competition experiment that compared the attraction potency between 2.5 µg and 7.5 µg of synthetic 6:1 mixture of (2*R*,3*S*) and (2*S*,3*S*) **13**. Two traps, containing 2.5 µg and 7.5 µg of **13** respectively, were placed to observe which trap do the males prefer. A total number of 11 males arrived at the 2.5 µg traps and 21 at the 7.5 µg traps. The number of males arrived at the two concentrations was not significantly different (Wilcoxon-test: Z = –1.64, P = 0.101, N = 25). Eight males moved from one trap to the other in the course of the observation period. This occurred in 6 cases from 2.5 to 7.5 µg traps and in 2 cases from 7.5 to 2.5 µg traps. Considering the ratio between the number of trials during which one or more males arrived at a trap and the occurrence of translocation, relatively more translocations occurred from the 2.5 to the 7.5 µg traps than vice versa (6 of 8 from 2.5 to 7.5 µg; 2 of 11 from 7.5 to 2.5 µg trap; Fisher exact test: P = 0.024), indicating that the traps with the higher concentrations of the test substance were more attractive to males.

In each of the eight trials more than one male arrived at a trap and in four of these aggressive encounters followed that led to the retreat of one male into the vicinity of the trap as would happen under natural situations involving a virgin female. Males also produced silken threads while moving from the wooden sticks to the vegetation and back in 21 cases.

As already discussed (section 3.12) each of the enantiomers might have a specific effect on the behavioral pattern of the spiders. While the synthesis of enantiopure trimethyl methylcitrate (**13**) was not successful, 2:1 and 6:1 diastereomeric mixtures of **13** were obtained**.** Another experiment was performed to compare the attraction power of these two diastereomeric mixtures which might at least partially indicate the influence of different stereoisomers. An amount of 7.5 µg each of a 6:1 and a 2:1 mixture were dissolved in 100µl dichloromethane and tested against each other. Males were attracted in 21 of 32 trials within an observation period of 30 minutes. In twelve cases the males contacted the 6:1 ratio trap and in nine cases the 2:1 trap, which is not significantly different (Binomial test: P=0.664). In total 29 males were attracted to the 6:1 ratio and 15 to the 2:1 ratio. This difference is not statistically significant (Wilcoxon-test: Z = –1.59, P = 0.111, N = 32). Eight males translocated from one trap to the other, five of which moved from 6:1 to 2:1 and three in the opposite direction. Two males moved from one trap to the other and back to the

previous trap (both directions). We conclude that the behavior of *A. bruennichi* males was not influenced by the diastereomeric mixture ratios offered.

3.16 Activity period in *Argiope bruennichi*

The pheromone release with time (in days) was determined using the CLSA technique (Section 3.7) with three different virgin female spiders during the month of July 2008. Female virgin spiders release the pheromone immediately after molting and gradually increase (Figure 30) their amount from day two onwards until day four, then start decreasing their emission until day eleven. No pheromone release was observed from day twelve onwards. The plotted graph with the values obtained from spider 1 is consistent with the values obtained from two other spiders as well.

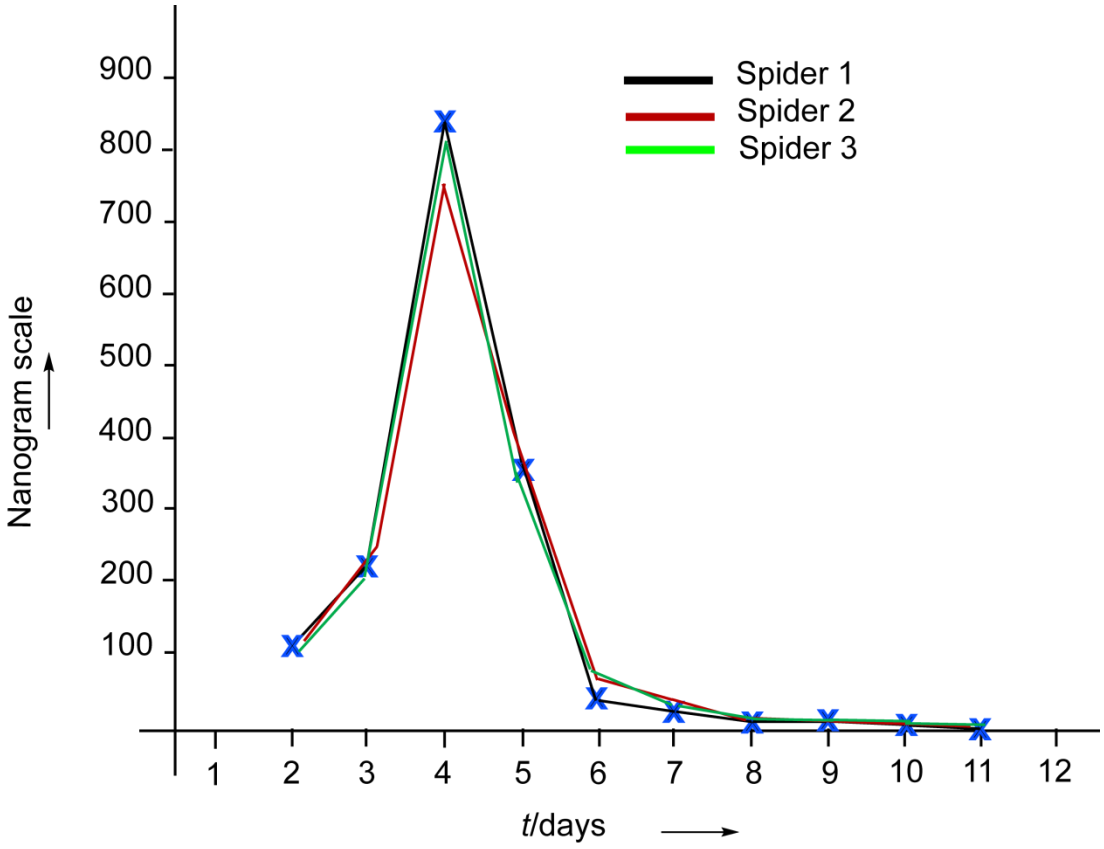


Figure 30. Pheromone release of freshly molted adult spider *Argiope bruennichi*

Samples were taken daily from the three different individuals (parallel CLSA experiments were performed) starting from day two to until day thirteen and the data (Table 4) obtained during GC-MS analysis was calibrated by using an internal standard of trimethyl citrate (**15**). No data was recorded on day 1 as all the three spiders were collected on day 1 after molting and CLSA experiments were performed for 24h. The data cannot be used as spider release rates per 24 h because it was not determined how the actual response of the system for **13** was. Errors can be introduced by adsorption on glass surface, trap capture, etc. Nevertheless, the general production of the pheromone can be established.

Table 4.Amount of pheromone released: Abundance (GC-MS intensity scale)

Time	Spider 1		Spider 2		Spider 3	
(in days)	Abundance	ng	Abundance	ng	Abundance	ng
1						
2	1.0×10^{7}	110	1.1×10^{7}	120	8.1×10^{6}	90
3	2.0×10^{7}	220	2.2×10^{7}	240	1.8×10^{7}	200
4	7.5×10^{7}	830	6.8×10^{7}	760	7.3×10^{7}	810
5	3.2×10^{7}	350	3.7×10^{7}	410	2.9×10^{7}	320
6	3.5×10^{6}	40	5.2×10^{6}	60	4.3×10^{6}	50
7	2.2×10^{6}	25	3.9×10^{6}	45	2.6×10^{6}	30
8	1.2×10^{6}	14	1.5×10^{6}	18	9.4×10^{5}	11
9	1.2×10^{6}	14	9.4×10^{5}	11	1.1×10^{6}	13
10	2.5×10^{5}	3	1.6×10^{5}	2	2.5×10^{5}	3
11	1.2×10^{5}	1,5	1.6×10^{5}	2	1.6×10^{5}	2
12	-	-	-	-	-	-
13	-	-	-	-	-	-

3.17 Biosynthetic proposal for the female specific compounds from *Argiope bruennichi*

The biosynthesis of 3-octanoyloxy-γ-butyrolactone (**14**) can be explained as shown (Figure 31 pathway **A**) starting from malic acid which is region-selectively reduced to form 3,4-dihydroxybutanoic acid (**34**) that later cyclises to give the hydroxybutyrolactone (**35**). Acylation of hydroxybutyrolactone (**35**) gives the final compound **14**.

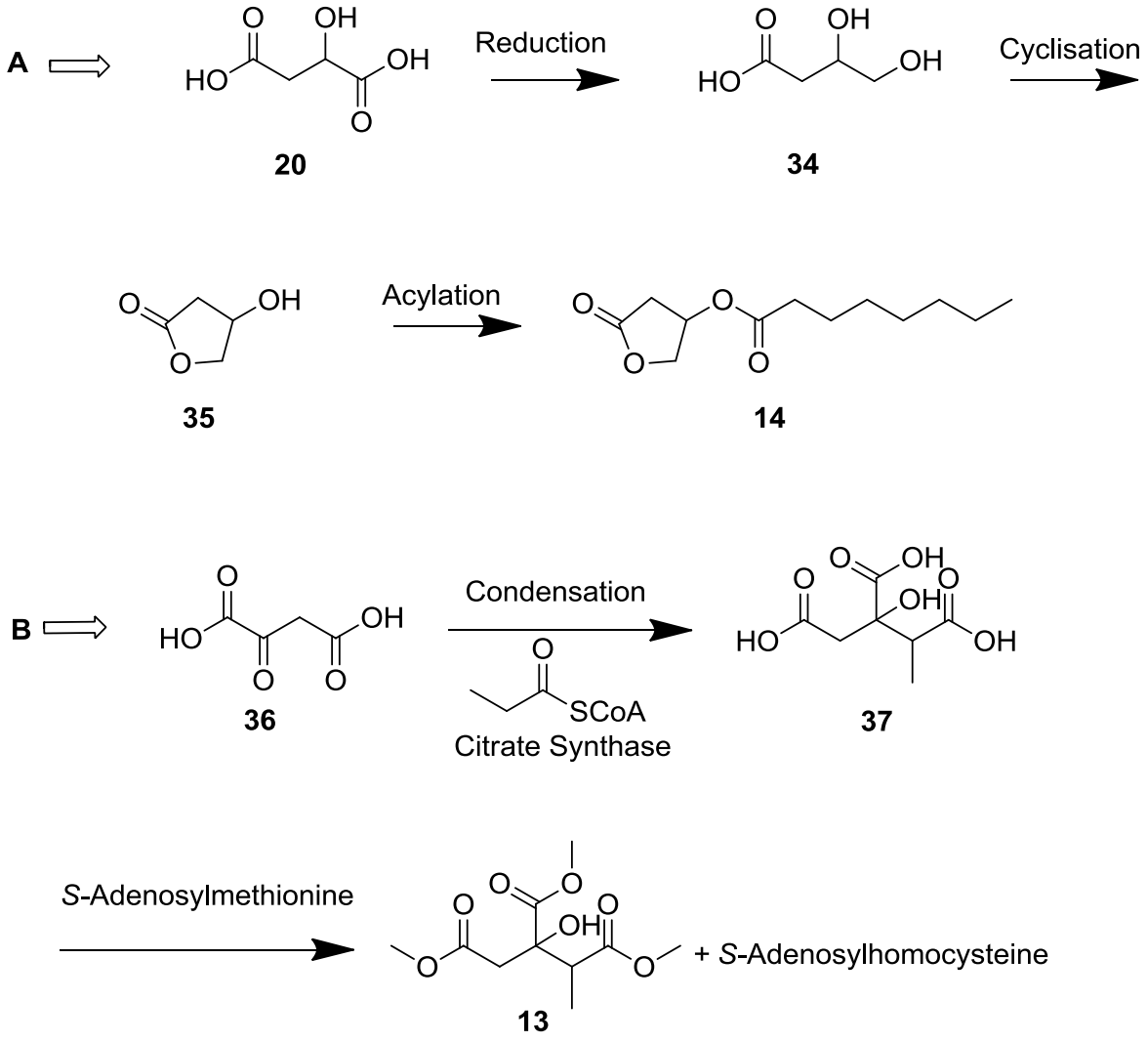


Figure 31. Proposed biosynthetic pathway **A**) 3-octanoyloxy-γ-butyrolactone (**14**) **B**) Trimethyl methylcitrate (**13**)

Whereas the biosynthesis of trimethyl methylcitrate (**13**) probably involves the condensation of propionyl-CoA and oxaloacetate (**36**) to methylcitric acid (**37**) catalyzed by the enzyme citrate synthase (Figure 31 pathway **B**), such as the *si-*

citrate synthase that normally catalyze the first step of the citric acid cycle in humans, or by the *re*-citrate synthase with a different stereoselectivity found in few anaerobic bacteria, or with still unknown selectivity, such as the enzyme methylcitrate synthase found in microorganisms.[82] Further methyl group transfer to the acid **37** furnishing **13** is catalyzed by *S*-adenosylmethionine (SAM).[85]

3.18 Conclusion

The sex pheromone of the wasp spider *Argiope bruennichi* has been identified as a mixture of 2*R*,3*S* and 2*S*,3*S* isomers of trimethyl methylcitrate (**13**) which was proven by synthesis. This molecule was able to attract male spiders in an open field via a trapping experiment performed which was never reported earlier till date. Activity of this molecule was concentration dependent and the presence minute quantities of the other isomers of trimethyl methylcitrate **13d & 13b** (2*S*,3*R* and 2*R*,3*R*) does not influence the behavior of male spiders.

The natural existence methylcitric acid in many organisms is a well known fact. For instance, 2*R*,3*S* and 2*S*,3*S* isomers are usually formed by the action of a *si*-citrate synthase in animals, such as pigs and humans,[82,83] whereas the other two isomers are formed by bacteria and yeasts using *re*-citrate synthase.[84]

The use of derivatives of citric acid, a typical primary metabolite, as pheromones has not been reported from other animals. Whether this close connection to primary metabolites is typical for pheromones of spiders will need further exploration. Another female specific spider compound, 3-octanoyloxy-γ-butyrolactone (**14**), has been identified, but its function needs to be established.

Trimethyl methylcitrate (**13**) was also identified from the web and headspace extracts of female *Argiope argentata* and *Argiope blanda* which suggests widespread presence of this molecule in the genus *Argiope*. Cross attraction within heterospecific species has not been investigated which might reveal the influence of species specific chemicals in the behavioral pattern.

4. Enantioselective synthesis of novel spider lipids from *Argyrodes elevatus*

4.1 Life style of *Argyrodes elevatus*

Spiders of the genus *Argyrodes* (Cobweb weavers) belonging to the family Theridiidae are usually small, cryptic in nature and are often found in the webs of the other spiders.[86] It is difficult to locate the members of this genus because of their hidden life style, form and behavior. Although these spiders are capable of forming webs occasionally, they prefer to invade the webs of other spiders (host) and lead a parasitic life. They can exist in a variety of relationships (kleptoparasite, commensal, web-stealer, host predator, scavenger) with the host spiders depending on factors such as relative size of the host, morphology of the host web, and host feeding rate.[87] Because of the large size[88] of the webbing provided by the host spiders (e.g. orb weavers) members of the *Argyrodes* family are easily able to invade them along several other factors are also favorable for the kleptoparasites as high host concentration, high food availability. Most of the *Argyrodes* species occur in the tropics and subtropics.[89]

The kleptoparasitic spider *Argyrodes elevatus* (Figure 32) commonly known as dewdrop spider (or silver spider) is found close to the webs of the orb weaving spiders *Argiope* or *Nephila* (Figure 33). It builds no webs for prey capture, instead it depends exclusively on the host web for obtaining food.[90]

Figure 32. The kleptoparasitic spider *Argyrodes elevatus* (Photo: G. Uhl.)

It patrols the frame web of the host *Argiope* picking off things that are caught. The spider treads very carefully as the owner of the web would easily eat the little dew-drop spider. Normally, the intruder is not detected by the host because the parasite is capable to move very carefully, but also fast in the web with its long front legs that wave forward and sideways during the raids. If prey is caught, the parasite moves to the prey and steals it before the host arrives.[90] However, the invasion on to the host web is not only beneficial to the invading spider itself (as it gains nutrition) but also advantageous to the host as well since it clears out the unwanted matter that might decay the web if remained on the web for longer periods.

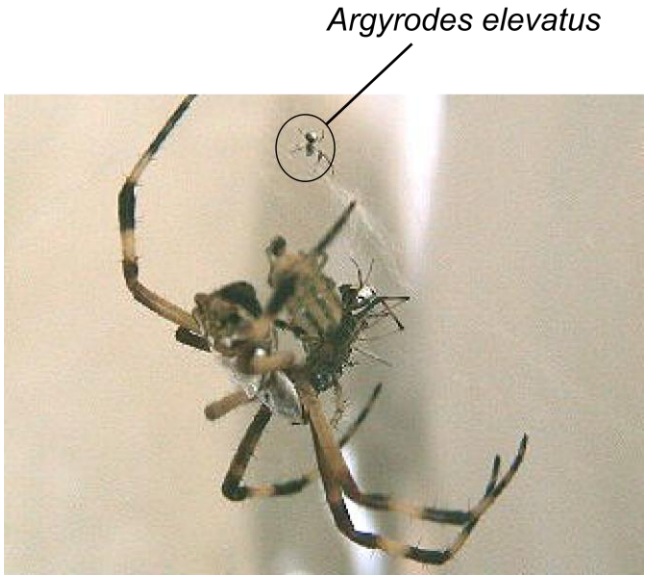


Figure 33. *Argyrodes elevatus* hanging in the web of *Argiope bruennichi* (Photo: G. Uhl.)

Spiders are normally pre-adapted for walking on silken threads and have highly developed vibratory receptors. *A.elevatus* sometimes hangs at the periphery of the host web with the help of finely laid signal threads which are connected to the radii of the host web. Vibrations from the prey wrapping movement are detected from the host and are transferred via these threads to the kleptoparasite. This process allows it to steal the food that is stored at the hub as in *Nephila clavipes*.[90]

A.elevatus on the webs of *Argiope* and *Nephila* is involved in three activities (i) removal of small insects ignored by the host (ii) share the food with host (iii) steal the food stored by the host.[91] Some *Argyrodes* however have been reported to feed on

the host or other spiders. For example *Argyrodes fissifrons* feeds on its host *Agelena limbata* during the molting period.[92]

Smaller orb-webs are a greater challenge for the parasite because its detection by the host is facilitated. Thus, the parasite must develop a more sophisticated strategy for the predation. The parasite cuts through the thread to the host web and bridges the gap with its body followed by coiling up the host web and simultaneously elongating the own thread. This process proceeds up to the detection of the prey and then the parasite snaps the item with its legs and falls off the host web.[93]

The density of *A.elevatus* invading the host web at a time might vary considerably with season and the host spider population. The number of individuals per web may go up to a maximum of 40-45 which might cause the host to search for another web site.[94,95] Occasionally several species of *Argyrodes* coexist in the same web occupying the different regions of the web and thus reducing the interspecific competition.[96]

4.2 Previously known spider lipids

Silk and cuticular lipid profiles of spiders predominantly consist of hydrocarbons which is common to several other arthropods.[97] The cuticular blend also essentially comprises of long chain aliphatic hydrocarbons and fatty acids with smaller quantities of waxy esters, long chain aliphatic alcohols, aldehydes, glycerides and cholesterol. Although the cuticle of spiders frequently serves as a source for pheromones, not much information is available on the composition of cuticular lipids and their involvement in spider communication.[30]

Earlier investigations on silk lipids of *Linyphia triangularis* revealed a new class of ether lipids (1-methoxyalkanes) (Figure 34) with a carbon chain length varying between 24 to 32 and methyl branches occurring predominately at C-2 position and ω-2 or ω-3 positions.[99] These methyl ethers were earlier not encountered in the cuticular hydrocarbons of insects.[100] Altogether, 27 different 1-methoxyalkanes were identified from *L. triangularis*. Similar or identical compounds were also found in other Linyphiids closely related to *L. triangularis* that showed species specific patterns.[99]

Existence of species specific mixtures of methylethers with relatively similar pattern of alkane mixtures might point out that they might be involved in species recognition.

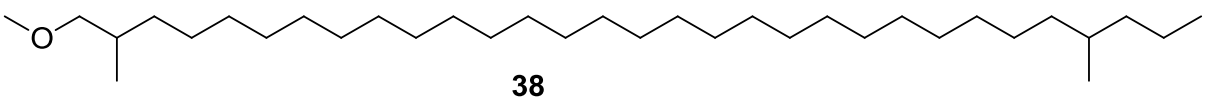


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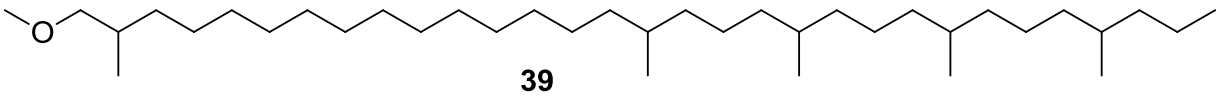
Figure 34. Branched alkyl methylethers identified in *Linyphia triangularis*

The silk and cuticular lipids of *Nephila clavipes* also comprised these unique class of compounds (Figure 35), consisting of straight chain and branched methyl ethers (1-methoxyalkanes).[98] They constituted 50-80% of silk lipids. The chain- length of these methyl ethers varied between 25 to 45 carbon atoms and branching up to four methyl groups occured. The composition also included 2-methyl branched hydrocarbons forming the second major class of compounds accompanied by small amounts of di, tri, and tetra methyl alkanes as well as *n*-alkanes. Several other classes of compounds like alkanols, alkanediols, fatty acids, and glyceryl ethers were also observed.[98]



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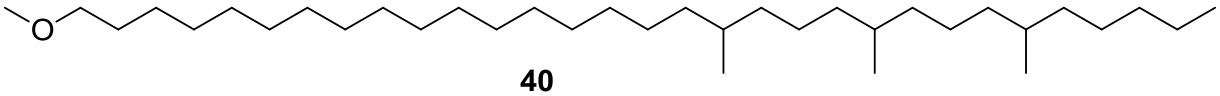

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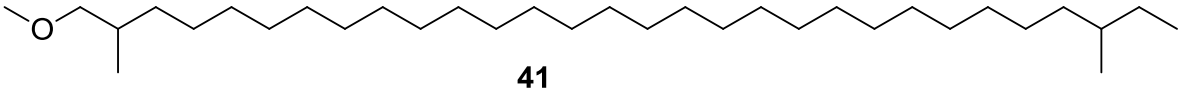

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Figure 35. Branched alkyl methylethers identified in *Nephila clavipes*

The 1-methoxyalkanes, identified as major group of lipids in *N.clavipes* (Figure 35) contain methyl branches located close to the alkane end of the molecule and are often arranged in a 1,5-pattern, with the exception of 1-methoxy-2,28-dimethyl triacontane (**41**) which is structurally similar to the methyl ethers of *L. triangularis*.

Small amounts of 1-methoxyalkanes also occurred in *Latrodectus revivensis.* Trials performed with silk of females containing unbranched 1-methoxy alkanes **42** released behavioral changes in males of *Latrodectus revivensis*. An additional methoxy group is present in some ethers from *Labulla thoracica*. 1-Methoxy-2-(methoxymethyl)-12-methyl nonacosane (**43**) and higher methylated homologs are found together with 1-methoxyalkanes on the lipid layer and silk of this species.[30,31]

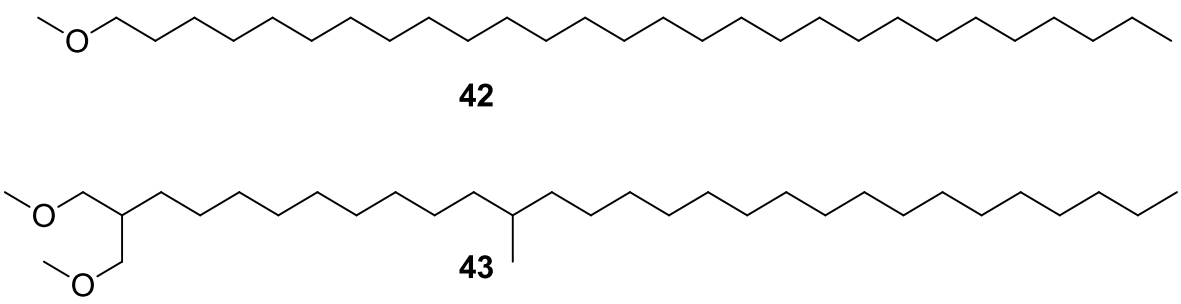


Figure 36. Methyl ethers from spiders. **42**: *Latrodectus revivensis*;
43: *Labulla thoracica.*

The lipid layer on the web of the daddy-long-leg spider *Pholcus phalangoides* consisted mainly (up to 90%) of the branched wax ester icosyl 2,4,6-trimethyltridecanoate (**44**), accompanied by low amounts of homologs.[101] Cuticular extracts of social spider *Anelosimus eximius* showed fatty acid methyl esters, hydrocarbons and novel long chain propyl esters **45**and **46** (Figure 37).[102]

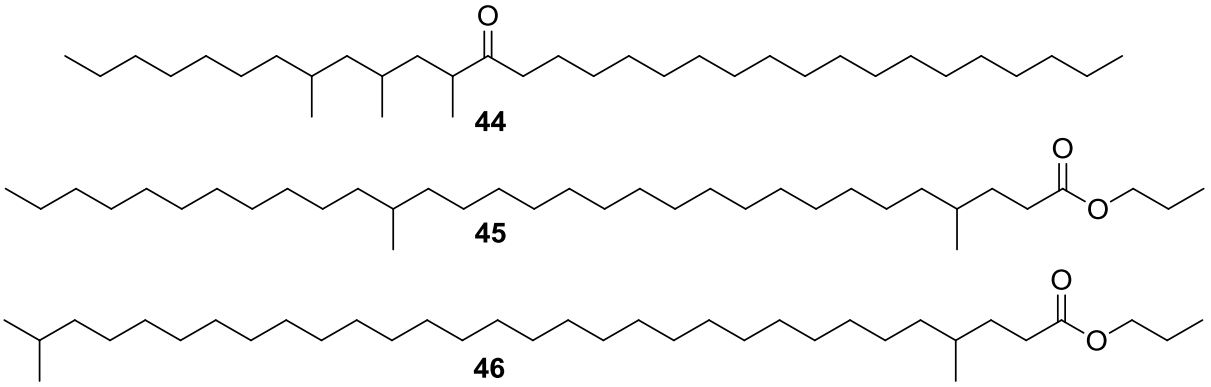


Figure 37. Esters from spiders. **44**: *Pholcus phalangoides*;
45, 46: *Anelosimus eximius*

These propyl esters (Figure 37) comprised almost three fourth of the composition with odd numbered carbon chain predominating and a maximum of three methyl branchings on even numbered carbons. The propyl esters 4,20- and 4,30-dimethylhentriacontanoate and propyl 6,20- and 6,30-dimethyl hentriacontanoate dominated the extracts.[102]

The absolute configuration of all the molecules illustrated above (**37-46** with the exception of **42**) remains unknown. Enantiomeric separation of these scarcely functionalized, long-chain methyl branched compounds on a chiral phase has yet to be designed. The primary function of these lipids on silk include water balance which in turn make the silk taut and gives it its elastic properties, thus protecting it against degradation. On the other hand they might also play a significant role in species recognition, and protecting the web against chemicals and microorganisms.[31,97,98]

4.3 Cuticular composition of male and female *Argyrodes elevatus*

Argyrodes is a kleptoparasitic spider living in the web of other species.Their cuticular composition is of intrest, because previous studies of related species showed the presence of signaling compounds. The head (prosoma) of male and female *A.elevatus* was soaked in dichloromethane and extracted. GC-MS analysis of these dichloromethane extracts showed the presence of a novel, unique class of compounds **47**-**51** (Figure 39) that were never identified earlier in spiders. Interestingly male and female extracts contained only a few number of distinct compounds. These compounds were identified as wax esters with a medium chain length in both parts. The appearance of methyl branches in the acid moieties and occasionally in the alcohol parts added structural diversity and significance to these compounds.

The gas chromatogram (Figure 38; top) from male extracts consisted of a single major compound **47** which contributed to 76% of the total ion chromatogram. This male specific compound was identified as C-25 ester containing branched C-13 acid and an unbranched C-11 alcohol. Methyl branching in acid part occurred at the C-2 position. Further derivatives of this compound class were present as trace amounts in the extracts along with the hydrocarbons which are typical to the lipid profile in arthropods.

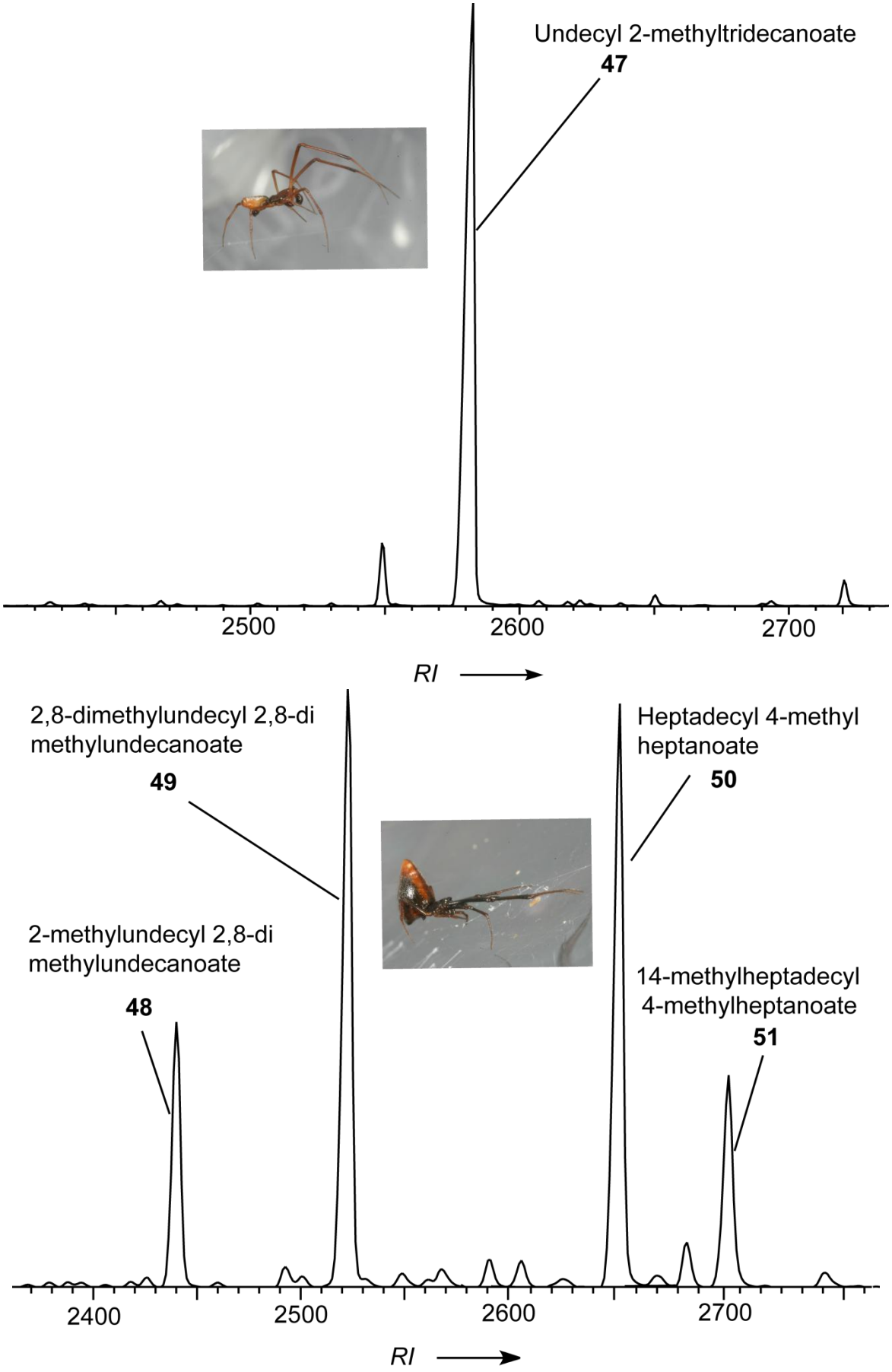


Figure 38. Total ion chromatogram of cuticular extract from *Argyrodes elevatus* Top: Male prosoma; Bottom: Female prosoma (Photo: G. Uhl.)

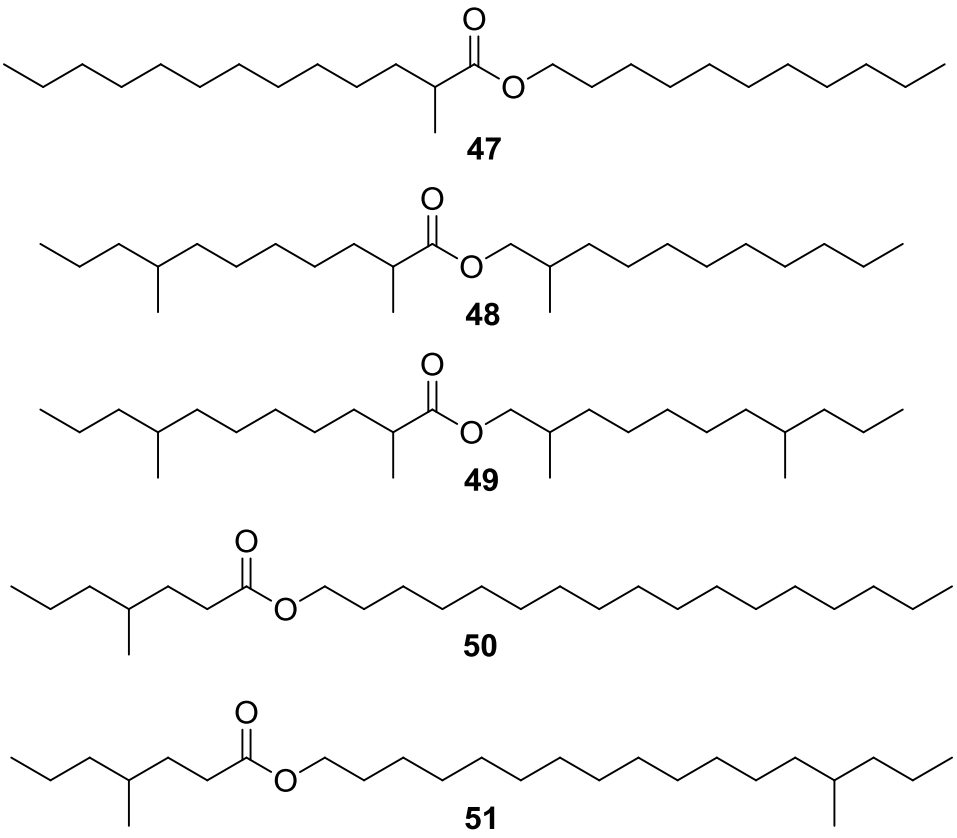


Figure 39. Cuticular components of *Argyrodes elevatus* **47**: Male; **48-51**: Female.

The gas chromatogram (Figure 38; bottom) belonging to female cuticular extracts preferentially showed four major peaks, also belonging to the class of long chain branched wax esters which corresponded to 75.8% of the total ion chromatogram. Female specific compounds were identified as 2-methylundecyl 2,8-dimethylundecanoate (**48**) (11.3%), 2,8-dimethylundecyl 2,8-dimethylundecanoate (**49**) (28%), heptadecyl 4-methylheptanoate (**50**) (26.2%), and 14-methylheptadecyl 4-methylheptanoate (**51**) (10.3%) as illustrated above (Figure 39). The cuticular extracts from the female prosoma also showed several other wax esters that occurred in minute quantities apart from the regular hydrocarbon blend.

Elucidation of the functional groups as well the position of the methyl branches was performed by analysis of mass spectra and use of gas chromatographic retention indices. Complexities caused by the internal positioning of the methyl branches were solved by microderivatizations such as transesterification, silylation, and esterification with 3-pyridinemethanol and nicotinic acid (Figure 40).[32]

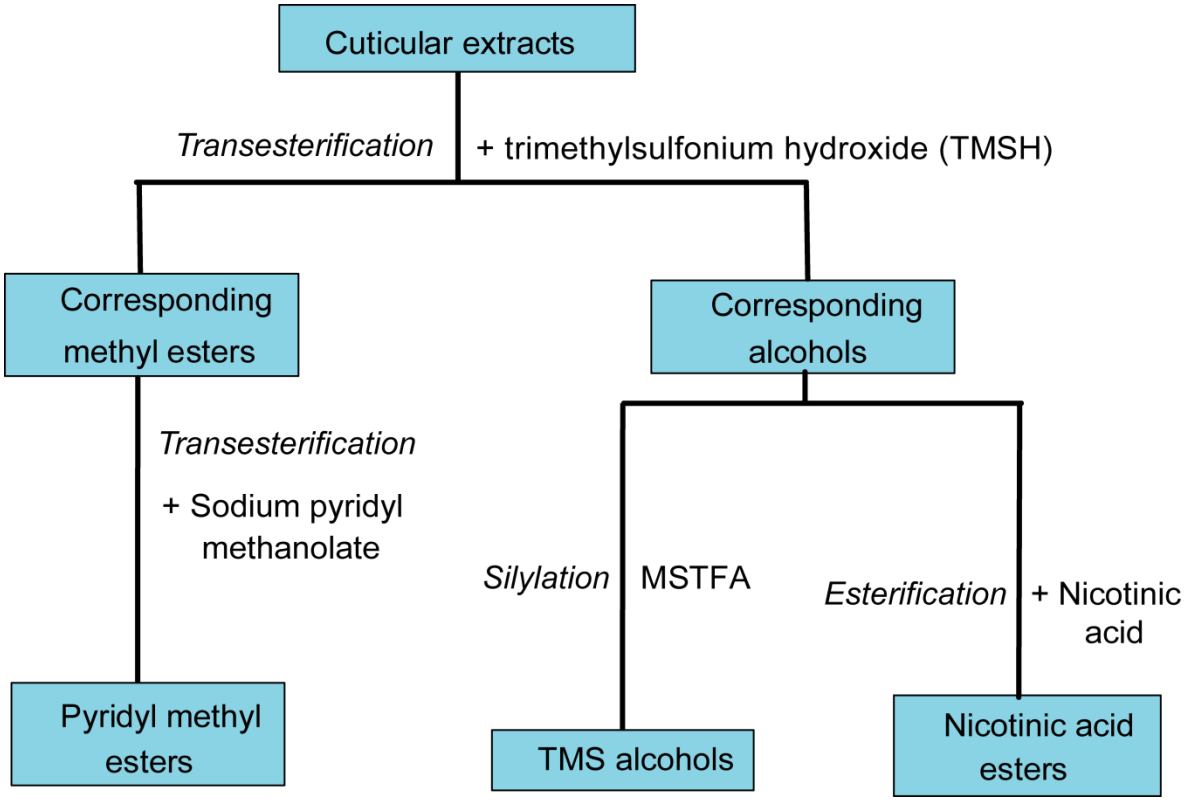


Figure 40.Flow scheme for microderivatizations of the wax esters from *A.elevatus*.

Treatment of the natural extract with trimethylsulfonium hydroxide cleaved the long chain esters into its corresponding acid and alcohol. Transesterification of the acid part with sodium pyridyl methanolate was instrumental in identifying the methyl branches positioned near to the ester functional groups (α-position) as well as internal branchings. Silylation of the corresponding alcohols gained from the natural extract proved methyl branches near to the silyl ethers functional group, on the other hand esterification with nicotinic acid was effective in establishing the internal position of the methyl branches.

Structure proposals, elucidations including derivatizations[32] of male and female specific compounds from *A.elevatus* were performed by Dr. Stephan Goller in the research group of Prof. Dr. Stefan Schulz while the confirmation of the proposed structures was fulfilled by a non stereo selective synthesis by me during my master thesis.[33]

4.4 Enantioselective synthesis of male specific cuticular compound

To clarify the stereochemistry of the natural compounds a enantioselective synthesis was performed (Figure 41) starting with tridecanoic acid (**52**) which was reacted with oxalylchloride giving the corresponding acid chloride (**53**).[103] The carbonyl derivative

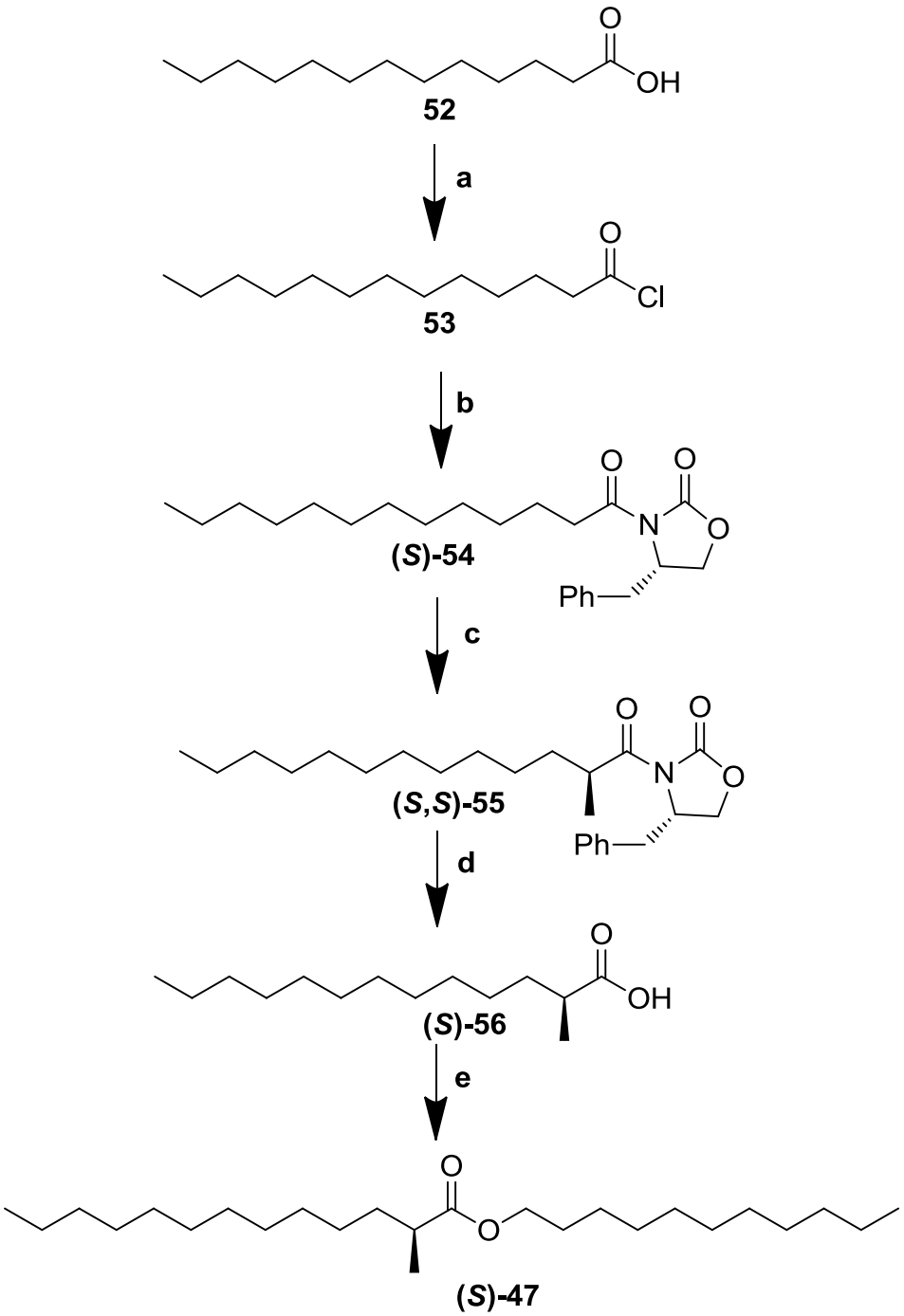


Figure 41. Synthesis of undecyl (*S*)-2-methyltridecanoate (***S*)-47 a**) $(COCl)_2$, 0°C, 91% **b**) (*S*)-4-benzyl-2-oxazolidinone, abs.THF, *n*-BuLi, -78°C, 30% **c**) Na-HMDS, abs THF, MeI, -78°C, 68% **d**) LiOH, 30% H_2O_2, THF:H_2O (2:1), 0°C, 69% **e**) EDC, undecanol, 49%.

53 was then treated with the Evans chiral auxiliary (*S*)-4-benzyl-2-oxazolidinone furnishing the chiral carboxylic acid derivative (***S***)**-54**.[104,105] Careful enolization of (***S***)**-54** with Na-HMDS at deep temperature, subsequently followed by the capture of the enolate with methyl electrophile (generated from methyl iodide) provided (*S*)-4-benzyl-3-((*S*)-2-methyltridecanoyl)oxazolidin-2-one ((***S***,***S***)**-55**) with diastereomeric ratio greater than 97% (GC value).[105] The chiral auxiliary employed imparts a high level of stereo-selection. The sodium enolate of (***S***)**-54** is preferentially attacked from one side only, thus leading to the diastereotopic discrimination.[106] The chiral auxiliary also allows the enolate to take the *cis* conformation which in turn directs the incoming electrophile to attack from the top side of the enolate.[80] Cleavage of the chiral auxiliary [107] from compound (***S***,***S***)**-55** was best done with LiOOH made from H_2O_2 and LiOH providing the α-methylated acid (***S***)**-56**. This hydrolysis step also allowed regaining the chiral auxiliary in pure form which was reused later. EDC esterification of the (*S*)-2-methyltridecanoic acid ((***S***)**-56**) with undecanol yielded the male specific wax ester (***S***)**-47**.[79]

4.5 Enantioselective synthesis of female specific cuticular compounds

The Initial steps in the enantioselective synthesis of one of the enantiomers from the female specific compounds followed a similar procedure (Figure 42) as described above. The chiral auxiliary which was regained by the hydrolysis of (***S***,***S***)**-55** was reused for formation of the chiral carboxylic acid derivative (***S***)**-59**[104,105] from pentanoyl chloride (**58**)**.** This was in turn obtained from the reaction of pentanoic acid (**57**) with oxalyl chloride.[103] Methylation of (***S***)**-59** using Na-HMDS gave (***S***,***S***)**-60** in a diastereomeric ratio greater than 97% (GC calculated value).[105] Cleavage of the chiral auxiliary from (***S***,***S***)**-60** by using LiOH and H_2O_2 afforded (*S*)-2-methyl pentanoic acid ((***S***)**-61**) in reasonably good yield.[107] Esterification of (***S***)**-61** with boron trifluoride and dry methanol[108] provided the corresponding methyl ester (***S***)**-62** which was reduced with $LiAlH_4$ into its respective alcohol (***S***)**-63**.[136] Treatment of (*S*)-2-methylpentan-1-ol ((***S***)**-63**) with bromine and PPh_3 yielded the corresponding bromide (***S***)**-64**.[109] Chain elongation proceeded via generating a Grignard reagent from (*S*)-1-bromo-2-methylpentane ((***S***)**-64**) which was coupled to 2-bromoethanol by the addition of dilithium tetrachlorocuprate, furnishing (*S*)-4-methylhepan-1-ol (***S***)**-65**.[110] Oxidation of (***S***)**-65** into its corresponding acid was achieved in two steps

which included initial conversion to the aldehyde[111] followed by transformation of aldehyde into the acid (***S***)-**66**.[112] Esterification of the acid (***S***)-**66** with heptadecan-1-ol using EDC gave one of the target female specific compound heptadecyl (*S*)-4-methylheptanoate ((***S***)-**50**)).[79]

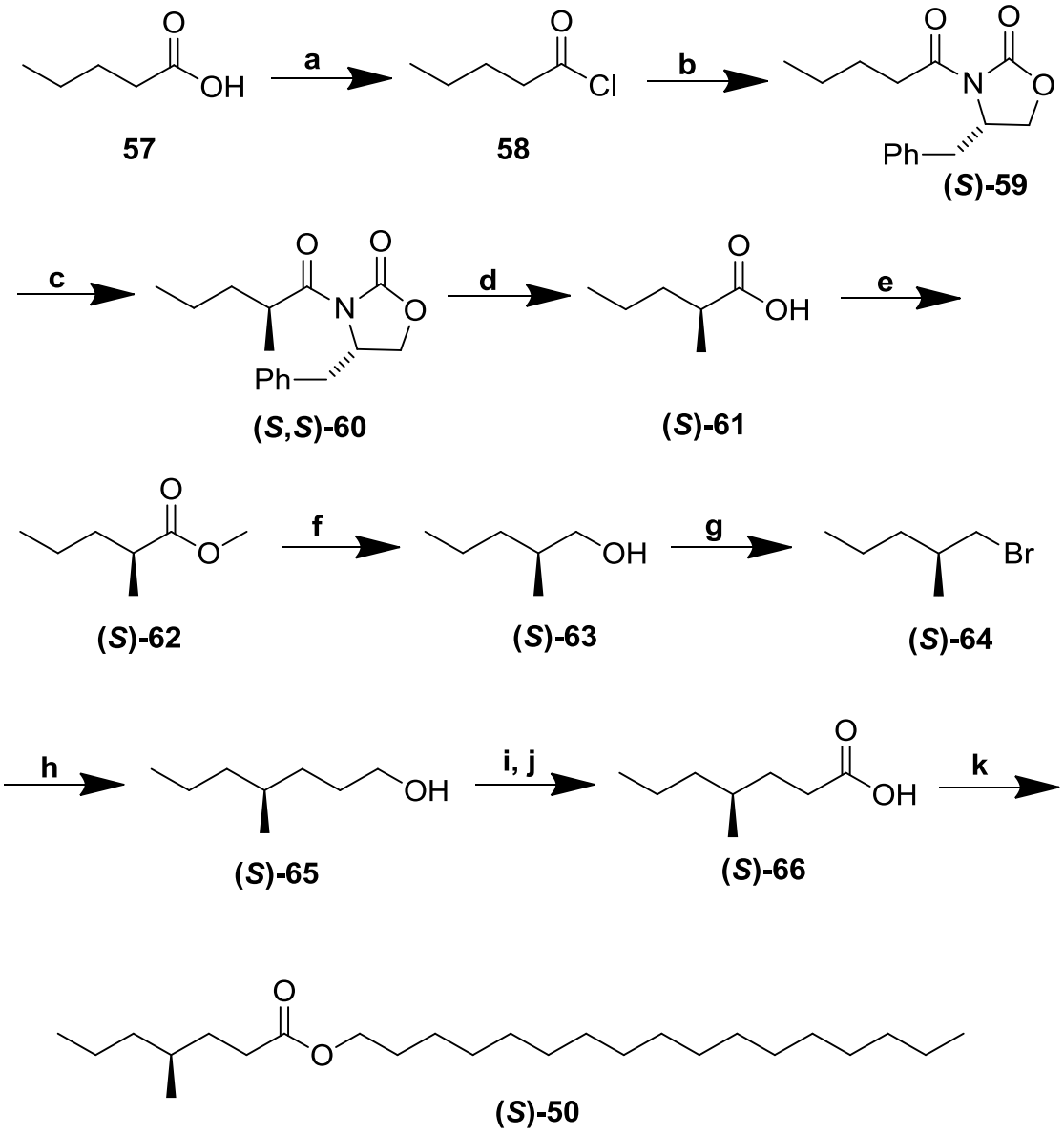


Figure 42. Synthesis of heptadecyl (*S*)-4-methylheptanoate (***S***)-**50** **a**) $(COCl)_2$, 0°C, 83% **b**) (*S*)-4-benzyl-2-oxazolidinone, abs.THF, *n*-BuLi, -78°C, 40% **c**) Na-HMDS, abs THF, MeI, -78°C, 92% **d**) LiOH, 30% H_2O_2, THF:H_2O (2:1), 0°C, 89% **e**) $BF_3{\cdot}(Et)_2O$, MeOH, 93% **f**) $LiAlH_4$, abs ether, 0°C, 98% **g**) PPh_3, Br_2, 0°C, 72% **h**) Mg, abs.THF, Li_2CuCl_4, 2-bromoethanol, 90% **i**) DMSO, $(COCl)_2$, $N(Et)_3$ 61%, **j**) $AgNO_3$, NaOH, 58% **k**) EDC, heptadecan-1-ol, 51%.

The synthesis of another enantiomer from female specific compounds used previously synthesized (*S*)-1-bromo-2-methylpentane ((***S***)**-64**) as starting material. This compound ((***S***)**-64**) was treated with magnesium turnings in dry THF to generate its respective Grignard reagent. Coupling of the Grignard reagent with 12-bromododecan-1-ol (**67**) by using dilithium tetrachlorocuprate[110] provided (*S*)-14-methylheptadecan-1-ol ((***S***)**-68**) which was finally reacted with previously produced (*S*)-4-methylheptanoic acid ((***S***)**-66**) by using EDC as catalyst, furnishing the target enantiomer (*S*)-14-methylheptadecyl-(*S*)-4-methylheptanoate ((***S***)-(***S***)**-51**).[79]

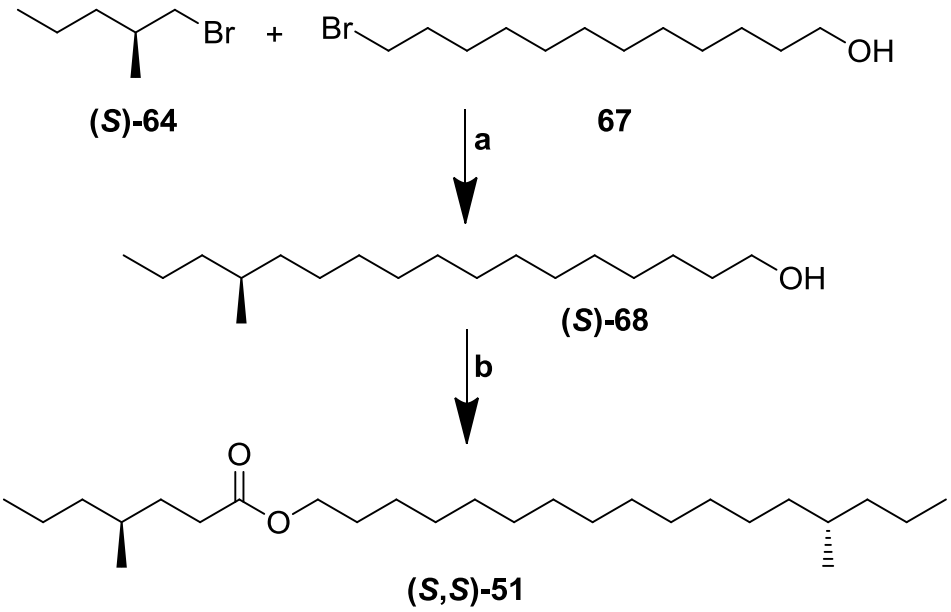


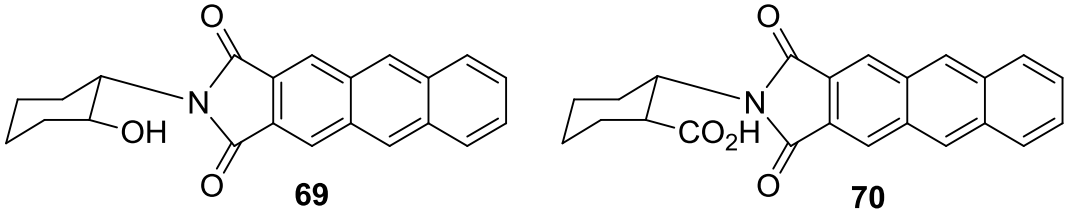
Figure 43. Synthesis of (*S*)-14-methylheptadecyl-(*S*)-4-methylheptanoate ((***S***,***S***)**-51**). **a**) Mg, abs.THF, Li_2CuCl_4, 21% **b**) EDC, (*S*)-4-methylheptanoic acid ((***S***)**-66**), 32%.

4.6 Chiral resolution

The possibility to determine the absolute configuration of the long chain wax esters with numerous stereogenic centers by comparing them with the synthetic samples which were obtained employing powerful enantioselective synthetic methods is quite complicated. It can be accomplished provided the stereoisomers can be separated so that analytical data of the natural product can be unambiguously compared with those of synthetic samples.

The major problem in separating the enantiomers by standard analytical methods such as GC and HPLC might prove difficult because of the carbon chain length of these esters. The difficulty is much increased due to the occurrence of stereogenic centers remote from the functional group. One possibility to solve this problem could be cleavage of the wax esters into the respective acid and the alcohols. Further transformation of the acid to methyl ester, results in relatively shorter chain methyl ester which can be employed as a reference sample. Applying similar procedure to the alcohol parts and as well to wax esters from the natural extracts, and co-injection on a chiral stationary phase in a GC instrument might prove fruitful.

Another possibility would be the use of fluorescent derivatizing reagents like (1*R*,2*R*)- and (1*S*,2*S*)-2-(2,3-anthracenedicarboximido)cyclohexanol (**69**) or cyclohexane carboxylic acid (**70**) which have been successfully applied in assigning the stereochemistry to long chain aliphatic natural products with remote functional groups. They serve as potential agents in determining enantiomeric purity of remote stereogenic centers even at concentrations up to 10^{-15} M.[113-115]



69 **70**

Figure 44. Structure of fluorescent derivatizing reagents for stereo-chemical analysis.

The above illustrated reagents (Figure 44) can be used to gain the respective acid or alcohol derivatives from natural extracts and synthesized enantiomers. A separation might be obtained in HPLC analysis at low temperature (-50°C).

4.7 Conclusion

The absolute configuration and biological roles of the wax esters **47**-**51** identified from male and female cuticular extracts of *A.elevatus* remained unknown. The position of methyl branches in these long chain wax esters was confirmed by microderivatizations along with non stereoselective synthesis. Applying efficient enantioselective methods and by employing Evans chiral auxiliaries three isomers have been synthesized out of 32 possible stereoisomers. Much more enantioselective synthesis has to be performed to assign the stereochemistry of the natural compounds and as well to study the relationship between the stereochemistry and specific activity of the long chain wax esters. Because no natural extract was available, no attempts for separation were performed.

The primary function of the cuticular lipid layer is the regulation of water content. The novel class of lipids on cuticle of *A.elevatus* might also serve as a source for short range pheromones because of their high molecular weight and non-volatile nature. These lipids might also function as tactile pheromones. Finally, these lipids may act as agents against microorganisms or degrading chemicals.

The existing sexual dimorphism in the blend of wax esters from male and female *A.elevatus* point out a significant function of these compounds. This information contained in the different composition of the lipids may be used for species recognition. The use of lipid layer in species recognition was reported earlier in *Frontinella pyramitela* on which the spider *Argyrodes trigonum* lives as a parasite, even in some cases feeding on the host itself.[116] Such cannibalistic behavior is observed only when the lipid layer is washed away with hexane.[117] From the above cited example we can also put forth an idea that the cuticular components of *A.elevatus* may also involve in discriminating the heterospecifics by chemical sensing or might also involve in host-specific recognition.

5. Analysis and synthesis of compounds identified in the body extracts of the sorghum chafer *Pachnoda interrupta*

5.1 Introduction on beetles

Beetles are insects and are classified into the order Coleoptera which is by far the largest order with most described species in the animal kingdom. It consists of 25% of the known life forms out of which 40% of the described insects are beetle species.[118]

Beetles are ubiquitous in distribution occurring in various size, shapes and colors making them a diversified group of insects. They are adapted to a variety of defense strategies to overcome the predator attack. These strategies include camouflage, mimicry or chemical defense. They often feed on all sorts of plant and animal material. Most of them are phytophagous, some are predaceous, some are scavengers, some feeding on mold and fungi with a few being parasitic in nature. The phytophagous species are free feeders on foliage, some pierce into the wood or fruit. They attack roots of the plant, while others feed on the parts of the blossoms, and some can even be leaf miners. To summarize, any part of the plant can be eaten up by some or the other type of beetle. Most of the beetles feed on broken plant and animal debris and organic material. Few of them also exist as commensals on the nests of social insects whereas some even feed on other invertebrates.[119]

The life cycle of the beetles is characterized by metamorphosis which starts after the adult female lays several dozens or even several thousands of eggs during its life time. The transformation from egg to the adult stage may vary from species to species. Four generations may be finished in one year while in other species four generation occurs in one year. Winter can be passed in any one of the life stages with relatively few species passing over winter as eggs. Larvae hatch from eggs and start to feed which is the principal feeding stage of beetle life cycle. The larval stage is the most voracious feeding stage that is supported by the presence of active chewing mouth parts and spiracles along the sides of the body. The larva develops into pupa from which the adult emerges having a varied life span ranging from weeks to years depending on the species.[119]

5.2 Taxonomy of beetles

One distinctive feature of Coleopterans is their wing structure. Most beetles have two pairs of wings. The front pair which may be thickened, leathery, or hard and brittle are called elytra that usually serve only as protective covers. A few beetles have greatly reduced wings. Variations in the structure of the first abdominal segment is one criterion used to separate the four various suborders of Coleoptera. The hind coxal leg segments (by which legs are attached to the body) may divide the abdominal segment partially or completely.[120]

- Suborder Archostemata - it includes about 40 species of small and medium sized beetles in five families. The larvae develop on fungus infested food where as the adults feed on the plant pollen which is suggested from its mouth parts.[121]
- Suborder Myxophaga – includes four families (approximately 100 species) of beetles that are relatively shorter in size (less than 2.5mm) which feed on algae or blue green algae.[121]
- Suborder Adephaga – includes 11 families with more than 40,000 species identified universally.[121] Most species of this suborder are predators with few exceptions in family Carabidae which is also the most diverse family in this suborder.[122]
- Suborder Polyphaga – this category containing 16 super families is the largest suborder containing over 300,000 known species arranged in more than 170 families. Among the commonly encountered polyphagans are the rove beetles (Staphylinoidea), scarabs and stag beetles (Scarabaeoidea), metallic wood-boring beetles (Buprestoidea), click beetles and fireflies (Elateroidea), as well as fungus beetles, grain beetles, ladybird beetles, darkling beetles, blister beetles, longhorn beetles, leaf beetles, and weevils.

5.3 Anatomy of beetles

The typical structure of beetle comprises of three main parts head, thorax and the abdomen like in any other insect (Figure 45). However, in Coleoptera two of the three segments (mesothorax and metathorax) of the thorax are attached to the abdomen,

while the third segment prothorax is isolated between head and trunk covered by a hard plate called pronotum. On the thorax three pair of legs and two pair of wings are situated. Beetles are also characterized by hard exoskeleton and hard fore wings called elytra which cover the hind part of the body to protect the second pair of wings. Elytra are not used for flight instead they have to be raised for the wings to operate.[119]

The antennae which are present on the head part are the most important sense organs along with the lobed pair of palps that may also detect smell. In some families the antennae are used for mating, and even for defense purposes. The mouth part which is used for chewing solid food comprises a pair of jaw like appendages called mandibles with blade like structures behind them called maxillae.[119]

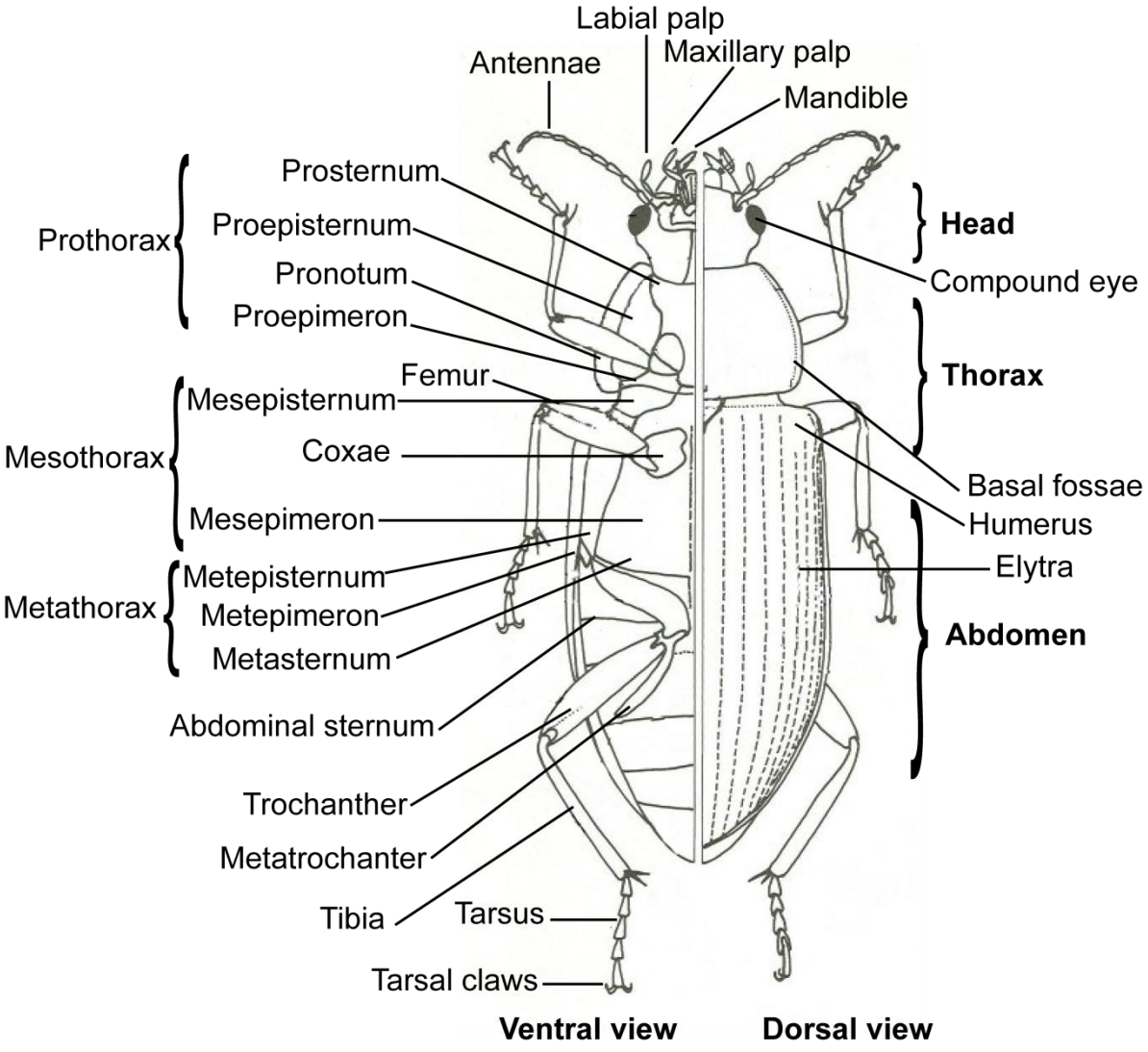


Figure 45. Structural anatomy of beetles (Photo: Scholtz and Holm)

Legs arising from thoracic segment are multi-segmented and are used for locomotion including swimming (last pair of legs modified for this purpose).[119] The jointed legs contain femur, tibia and tarsus. The end of five segmented tarsi bears tarsal claws.

5.4 Beetle species as pests and benefits

Beetles can be beneficiary or injurious causing destruction to the human kind. Largely they are pests in agriculture resulting in reduction or loss of food-plant yield. The beetle *Dendroctonus ponderosae* belonging to the family Curculionidae, pierces the barks of several pine trees in regions of Northern America causing the death of the trees.[122] The Coleopteran beetle *Leptinotarsa decemlineata* is a serious pest on potato plants, tomatoes and eggplants.[123,124] Both the adult and larvae of the Japanese beetle *Popillia japonica* feed on multiple host plants causing damage to foliage, flowers and roots.[125] Pine and spruce trees of all ages are attacked by *Ips* bark beetles, of which the species *Ips hunteri* attacks spruce trees around Colorado, whereas *Ips calligraphus* attacks pine trees.[126]

The bark beetles *Hylurgopinus rufipes* and *Scolytus multistriatus* attack elm trees. The European elm bark beetle *S. multistriatus* and the large elm bark beetle *S. scolytus*, which like the American elm bark beetle *Hylurgopinus rufipes* transmit Dutch elm disease fungi. The bark beetles carry Dutch elm disease as by moving from infected breeding sites to feed on healthy elm trees. The spread of the fungus by the beetle has led to the devastation of elm trees in many parts of the Northern Hemisphere, notably in Europe and North America.[127] Flea beetles are common pests of many vegetable crops like corn, cabbage and lettuce.

Other than causing serious destruction to agriculture and horticulture, beetles can also be beneficiary which is mainly derived form their feeding habits. Dung beetles belonging to the family Scarabaeidae successfully reduce the populations of pestilent flies and parasitic worms that breed in cow dung.[128] Ground beetles which can not fly remain on the surface of the soil and feed on several invertebrates that cause damage to plants. Lady beetles (Coleoptera:Coccinellidae) are among the best known predatory insects that feed primarily on aphids. They actively feed in both larval and adult stages.[129]

5.5 Case history of *Pachnoda interrupta*

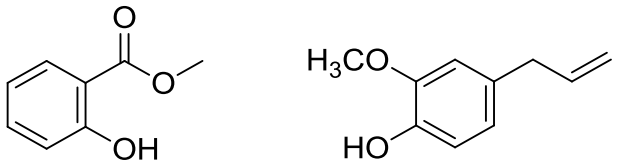
The beetle *Pachnoda interrupta* (Coleoptera:Scarabaeidae) commonly called as sorghum chafer as it is a key pest on *Sorghum bicolor* in Ethiopia that accounts for 70% of crop loss every year. Adults of *P. interrupta* are polyphagous herbivores feeding on fruits and flowers of several plant species that include banana, mango, acacia, orange, and papaya.[130,131] They also actively feed on *Abutilon figarianum* (herbaceous weed) and some food crops such as *Pennisetum glaucum* (pearl millet).[132] They mate and feed in July, while in October, the newly emerged adults feed only before going into aestivation until July of the following year.

Figure 46. Picture showing *P. interrupta* (Photo Left: Massimo forti Right: U.Schmidt, 2006)

These beetles are highly attracted towards a native Ethiopian beer like beverage called Tella (Ministry of Agricultural and Ethiopian Agricultural Research Organization 1999) which is a spontaneously fermented beer (without yeast) brewed using water, sorghum flour, cereals, malt of barley or wheat and crushed leaves of *Rhamnus prinoides*.[133]

After the early years of 1990 during which *P. interrupta* has emerged as a key pest in Ethiopia, lack of efficient control methods led to develop various methodologies in finding attractants for pest insects. Initially trapping the organisms by using fruit sources (especially banana) as bait showed promise up to certain extent (Ministry of Agricultural and Ethiopian Agricultural Research Organization 1999).

Previous field experiments which revealed that common compounds identified in the odor profile of fruits and flowers have the potential to attract masses of *P. interrupta.* The screening of the compounds was done by GC-EAD which allowed the identification of antennally active compounds in the volatile blend emitted by the host (sorghum). The attraction levels were highest with single compound lures that contained synthetic methyl salicylate (**71**) and eugenol (**72**).[134]

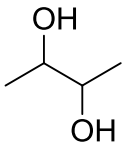


71
Methyl salicylate

72
Eugenol

Figure 47. Host specific compounds attracting the sorghum chafer.

More recent investigations employing GC-EAD studies on volatile collections from sorghum and the highly attractive weed *Abutilon figarianum* proved that the attraction is governed by a few influential compounds, rather than specific odor blends. Traps performed with synthetic sorghum and *A. figarianum* odor blends showed that racemic 2,3-butanediol (**73**) is a powerful novel attractant for *P. interrupta*. Although the blend from sorghum and *A. figarianum* were attractive, neither of them were more attractive in comparison to the single lure attractions with **71** and **72** in spite of which the latter were also part of *abutilon* blend.[133]



73
2,3-butanediol

Figure 48. Specific attractant of *P. interrupta* from odor blend of sorghum and *Abutilon figarianum*

The above results clearly suggest these semiochemicals can be potentially used in controlling sorghum chafer and monitoring it either by mass trapping and/or as part of an integrated pest management programme. Therefore a project was started to investigate whether *P. interrupta* pheromones could be used as attractants.

5.6 Collection of extracts from *Pachnoda interrupta*

Adults of the beetle *P. interrupta* form aggregations during the time of mating in July and October. During the mating season, field traps performed with live beetles demonstrated the attraction of males and females to unmated females indicating the existence of a female-emitted pheromone. Unmated females combined with a food source (mostly banana) attracted high number of male and female beetles. Other combinations with a food source including attraction to mated females and males were insignificant. This aggregating behavior pointed out that the attraction might be caused from a combination of host volatiles along with a female emitted pheromone.

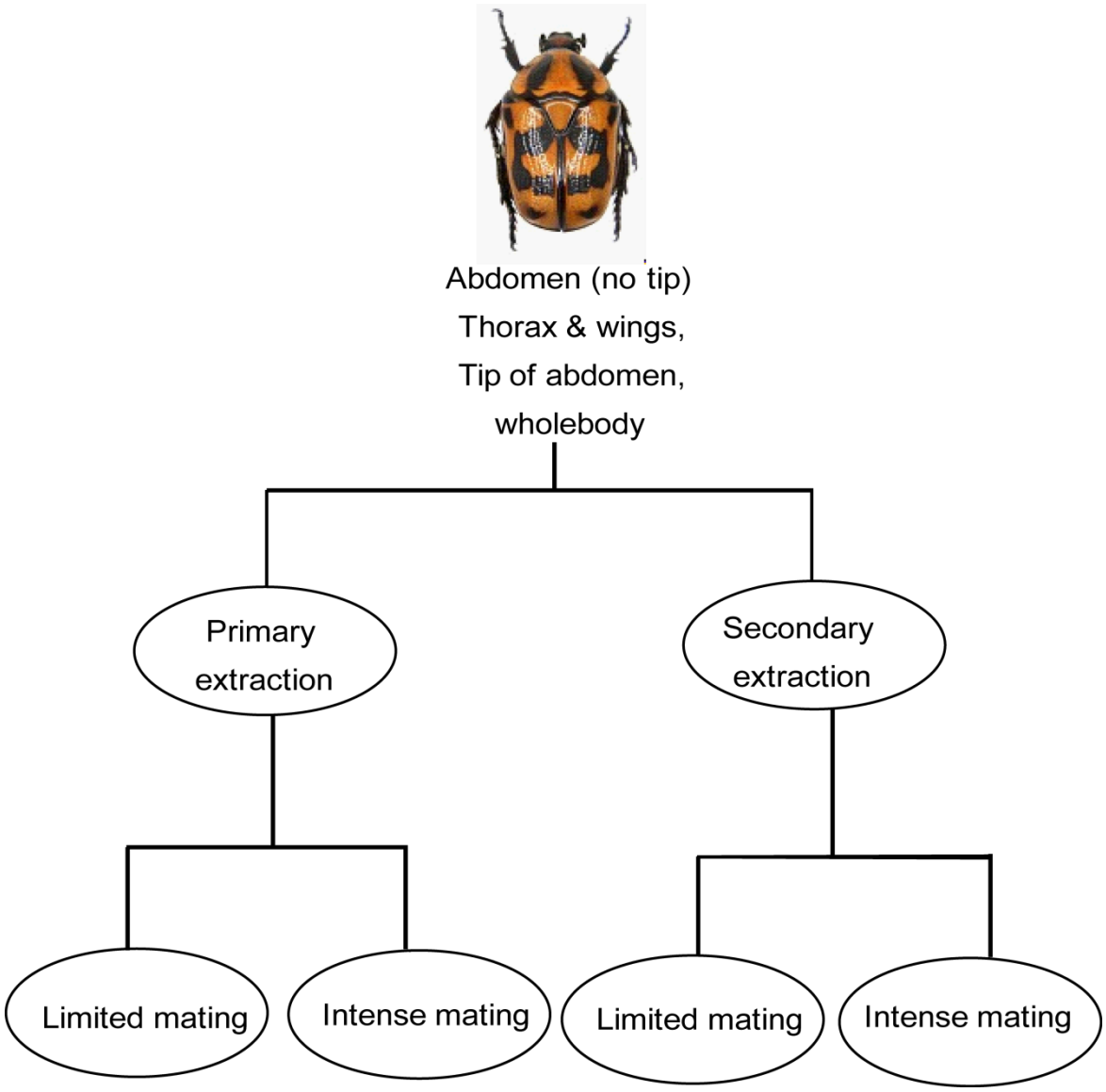


Figure 49. Schematic representation of hexane extracts collection from *Pachnoda interrupta*

In order to investigate the possible pheromone compound from *P. interrupta*, male and female beetles were excavated from aestivation sites in the field. They were separated according to the sex and stored under normal conditions of light and temperature. Extractions were performed by putting a male and female together in box. When the male started clasping the female for mating, they were separated and put in an Erlenmeyer flask. An identical procedure was followed until ten beetles were added to the flask, after which redistilled hexane (approximately 5ml) was added covering the surface layer of the beetles. Slight agitation of all the beetle samples for five minutes, followed by collecting the solvent, gave primary extracts that were investigated using GC-MS. The same beetle samples were treated again with hexane, and allowed them to stand overnight in case any compound that did not get dissolved into hexane during primary extraction. The solvent collected after this period was named as secondary extract. Extractions with whole body as well as extractions with different body parts that included tip of the abdomen, wings and thorax, abdomen without tip were also performed in a similar manner as described above.

The obtained extracts were collected in two different mating states (Figure 49). Intense mating stage, when previously separate beetles were put together, male beetles immediately started to attempt to copulate with females and limited mating state when there was a little delay in males responding to copulate with females. The hexane extracts were obtained from ten different beetle samples in each case and condensed by evaporating at room temperature if needed. This entire collection and extraction work was performed by Jonas M. Bengtsson from Swedish University of Agricultural Sciences, Alnarp who was a co-worker in this project.

5.7 GC-MS investigation of extracts from male and female *Pachnoda interrupta*

The male and female beetle extracts obtained by different extraction procedures with hexane were analyzed by GC-MS.

5.7.1 Composition of extracts from primary limited mating state of females

Gas chromatograms resulting from primary female extracts obtained during the limited mating stage was dominated by hydrocarbons and fatty acids which are the typical constituents of insect cuticle and fat body. Apart from this, trace concentrations of volatile compounds were also identified. Several monoterpenes, namely α-pinene (**74**), β-pinene (**75**), p-cymene (**76**), and limonene (**77**) along with geranylacetone (**78**) occured (Figure 50) in the extracts from all parts of the body (Table 5) which were not observed in any of the male extracts (Table 8 & 9). Identification of the exact isomer of any of these compounds was not possible because of their low concentrations. Apart from above mentioned compounds two other compounds tricosa-2,4-diene (**79**) and pentacosa-2,4-diene (**80)** were also identified only in all female beetle extracts.

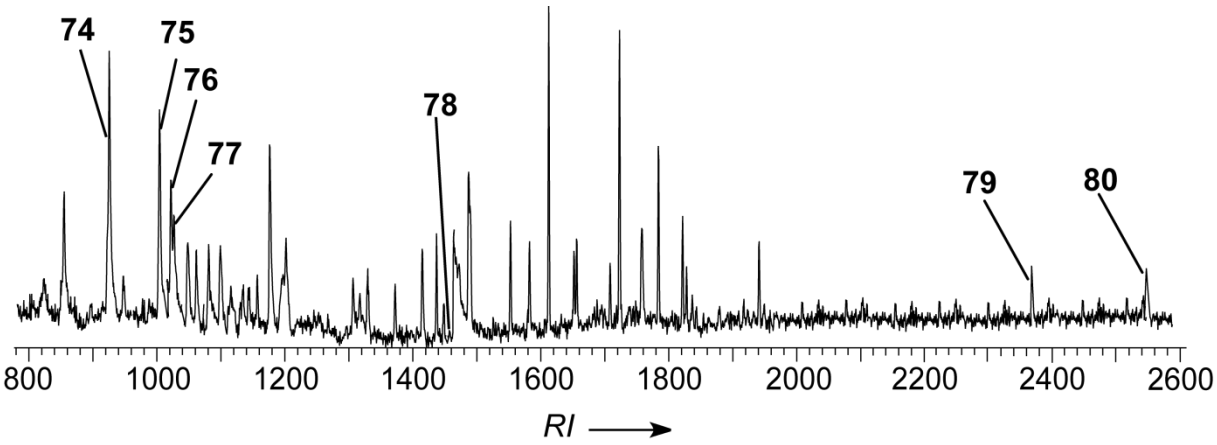


Figure 50. Gas chromatogram from the abdominal tip of primary limited mating state of female *Pachnoda interrupta*

Table 5. Composition of female primary mating state (A-Abdomen; TOA-Tip of the abdomen; WB-whole body; W&T- Wings and thorax; present (+); absent (-))

RI	Compounds	A	TOA	WB	W&T
930	α-Pinene*	+	+	+	+
1006	β-Pinene *	+	+	+	+
1025	p-Cymene	+	+	+	+
1027	Limonene*	+	+	+	+
1105	Nonanal	-	-	-	+

RI	Compounds	A	TOA	WB	W&T
1121	Methyl octanoate	-	-	+	+
1329	Methyl decanoate	-	+	+	+
1308	Undecanal	+	-	-	-
1461	Geranylacetone	+	+	+	+
1782	Tetradecanoic acid	-	+	-	+
1885	Pentadecanoic acid	-	+	-	-
1981	Hexadecanoic acid	-	+	-	+
2179	Octadecanoic acid	+	-	+	+
2374	Tricosa-2,4-diene	+	+	+	+
2577	Pentacosa-2,4-diene	+	+	+	+
2686	Pentacos-1-ene	+	+	-	-
2826	Squalene	+	+	+	+

*Absolute configuration not determined

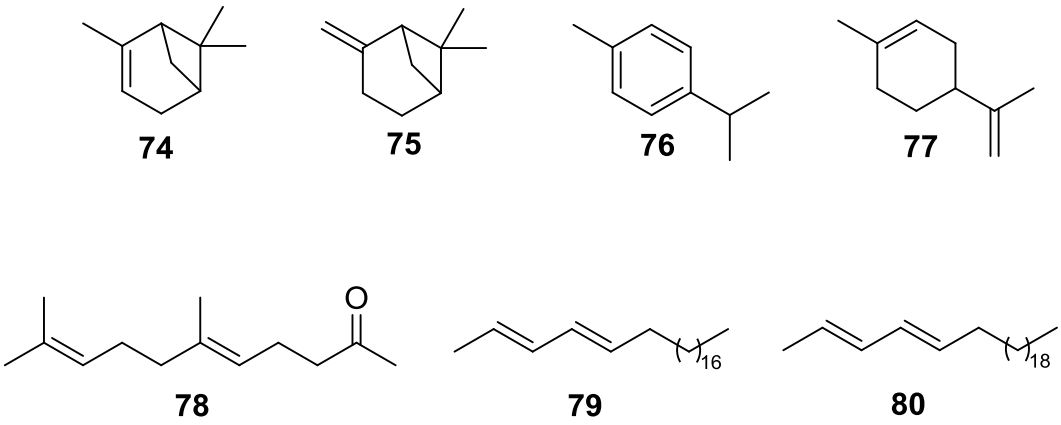


Figure 51. Structures of compounds identified in primary limited mating state of female *Pachnoda interrupta*

5.7.2 Composition of extracts from primary intense mating state of females

Investigation of extracts collected from primary intense mating state also showed compounds **74-80** along with hydrocarbons and fatty acids (Table 6), but aldehydes and geranylacetone (**78**) identified in the primary limited mating state were absent.

Table 6. Composition of primary female intense mating state (A-Abdomen; TOA-Tip of the abdomen; WB-whole body; W&T- Wings and thorax; present (+); absent (-))

RI	Compounds	A	TOA	WB	W&T
930	α-Pinene*	+	+	+	+
1006	β-Pinene *	+	+	+	+
1025	p-Cymene	+	+	+	+
1027	Limonene*	+	+	+	+
1121	Methyl octanoate	-	-	+	+
1329	Methyl decanoate	-	-	+	+
1674	Heptadec-1-ene	+	-	+	+
1862	Nonadec-1-ene	-	-	+	-
1928	Methyl hexadecanoate	-	-	-	+
1981	Hexadecanoic acid	-	-	-	+
1994	Ethyl hexadecanoate	+	-	-	-
2063	Henicos-1-ene	-	+	+	+
2270	Tricos-1-ene	-	-	+	+
2374	Tricosa-2,4-diene	+	+	+	+
2410	Icosan-1-ol	+	+	-	-
2468	Pentacos-1-ene	-	+	+	+
2569	Hexacos-1-ene	-	+	+	+
2577	Pentacosa-2,4-diene	+	+	+	+
2671	Heptacos-1-ene	+	+	+	+
2826	Squalene	+	+	+	+

*Absolute configuration not determined

5.7.3 Composition of extracts from secondary limited and intense mating state of females

The limited and intense female secondary extracts showed fourteen additional female specific compounds **81-94** (Figure 52 & 53) along with monoterpenes **74-77**, geranylacetone (**78**) and conjugated dienes **79** and **80** (Figure 50 & 51). The identified compounds were short chain carboxylic acids, esters, aromatic compounds and aldehydes (Table 7). Indole (**91**), propyl acetate (**81**), and 2-methylpropyl acetate (**83**) were found in larger quantity, up to 1.3 ng per individual (Table 8).

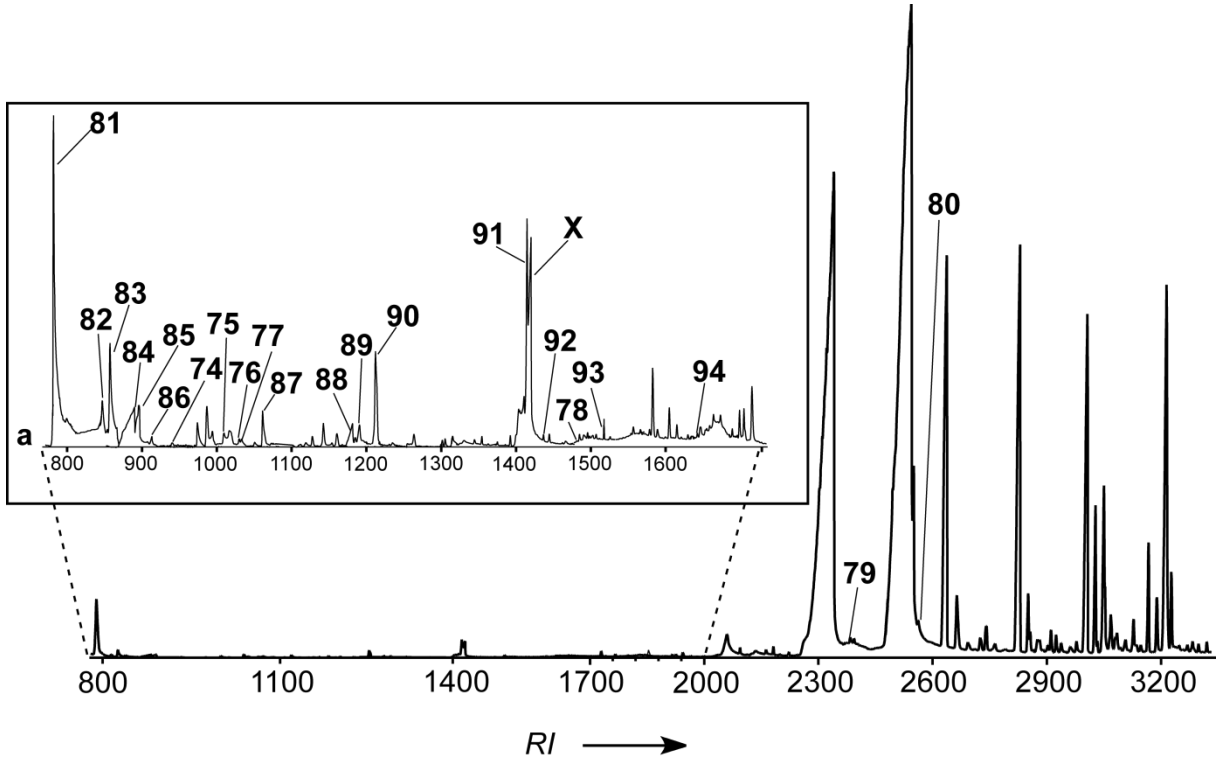


Figure 52. Gas chromatogram of the extract from abdominal tip of the female *P. interrupta* : secondary intense mating

a)Magnified chromatogram showing female specific compounds **81**. Propylacetate **82**.1-Methylpropyl acetate **83**. 2-Methylpropyl acetate **84**. 3-Methylbutyric acid **85**. 2-Methylbutyric acid **86**. Hexanoic acid **74**. α-Pinene **75**. β-Pinene **76**. p-Cymene **77**. Limonene **87.** Phenyacetaldehyde **88**. Benzoic acid **89**. Octanoic acid **90**. 2-Decenal **91**. Indole **92**. 2-Undecenal **78**. Geranylacetone **93**. (2*E*,4*E*) - Dodecadienal **94**. 4-Dodecanolide. **79**. Tricosa-2,4-diene **80**. Pentacosa-2,4-diene **X** . Artifact

Table 7. Composition of female secondary limited and intense mating states (A-Abdomen; TOA-Tip of the abdomen; WB-whole body; W&T- Wings and thorax; present (+); absent (-))

RI	Compounds	A	TOA	WB	W&T
775	Propyl acetate	-	+	-	-
865	1-Methylpropyl acetate	-	+	-	-
869	2-Methylpropyl acetate	-	+	-	-
893	3-Methylbutyric acid	-	+	-	-
897	2-Methylbutyric acid	-	+	-	-
912	Hexanoic acid	-	+	-	-
930	α-Pinene*	+	+	+	+
1006	β-Pinene *	+	+	+	+
1025	p-Cymene	+	+	+	+

RI	Compounds	A	TOA	WB	W&T
1027	Limonene*	+	+	+	+
1039	Phenylacetaldehyde	-	+	-	-
1105	Nonanal	+	+	+	+
1121	Methyl octanoate	+	-	+	+
1180	Benzoic acid	-	+	-	-
1189	Octanoic acid	-	+	-	-
1190	Ethyl octanoate	+	-	-	-
1206	(*E*)-Decenal	+	+	-	+
1329	Methyl decanoate	-	+	+	+
1380	Decanoi cacid	-	+	-	-
1423	Indole	-	+	+	-
1430	(*E*)-2-Undecanal	-	+	-	-
1461	Geranylacetone	-	+	-	-
1511	(2*E*,4*E*)-2,4-Dodecadienal	-	+	-	-
1481	Undecanoic acid	-	+	-	-
1570	Hexadec-1-ene	+	+	+	+
1585	Dodecanoic acid	+	+	+	-
1594	Ethyl dodecanoate	+	+	-	+
1653	4-Dodecanolide	-	+	-	-
1674	Heptadec-1-ene	-	-	-	+
1782	Tetradecanoic acid	+	+	+	-
1794	Ethyl tetradecanoate	+	+	+	+
1904	Heptadecan-2-one	-	-	+	-
1928	Methyl hexadecanoate	+	-	-	+
1976	Ethyl hexadecenoate	-	-	-	+
1994	Ethyl hexadecanoate	+	+	+	+
2080	Heptadecanoic acid	-	+	-	-
2167	Octadecenoic acid	+	+	+	+
2179	Octadecanoic acid	+	+	+	+
2374	Tricosa-2,4-diene*	+	+	+	+
2410	Icosan-1-ol	-	+	+	-
2468	Pentacos-1-ene	-	+	-	-
2506	Henicosan-1-ol	-	+	-	-
2569	Hexacos-1-ene	-	+	-	-
2577	Pentacosa-2,4-diene*	+	+	+	+
2671	Heptacos-1-ene	+	-	+	+
2826	Squalene	+	+	+	+

*Absolute configuration not determined

Although compounds **74-80** were identified in the primary extracts the additional fourteen female specific compounds **81-94** could only be found in secondary extracts. One possible reason for this observation could be that the first short-time extraction with hexane only removed compounds present on the outermost cuticle, or the surface of the insect body. Prolonged extraction allowed the hexane to penetrate

deeper inside the cuticle and body tissue and thus eluted the fourteen additional compounds **81-94.** Identification of compounds was performed by comparison of mass spectra and gas chromatographic retention indices with those of synthetic reference compounds.

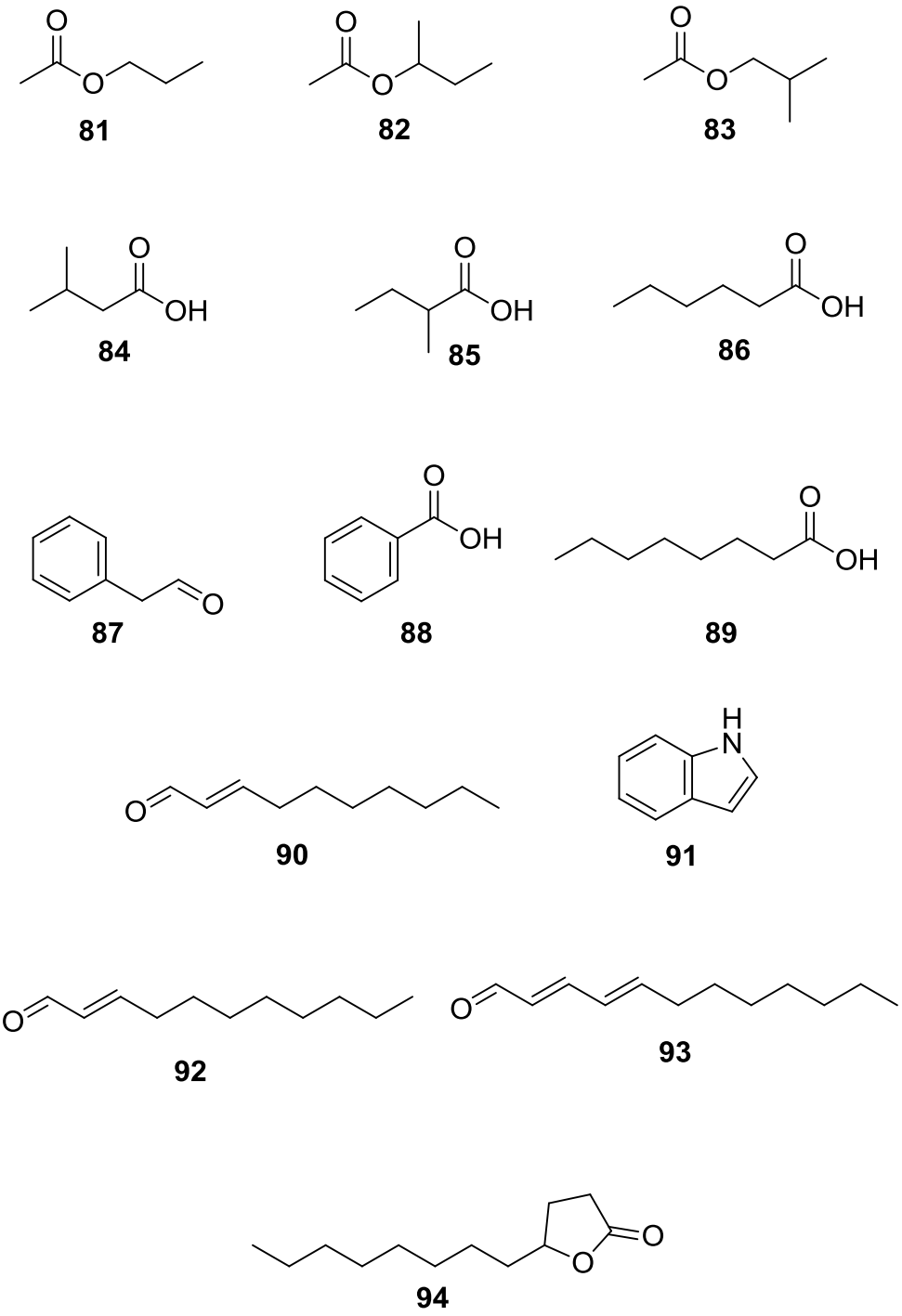


Figure 53. Structure of fourteen additional compounds identified in secondary limited/intense mating state of female *Pachnoda interrupta*

The concentration of female specific compounds **74-94** was estimated (Table 8) by using commercially available nonanal as internal standard. The concentrations estimated were in accordance with the extracts of secondary limited/intense mating stage. Extracts from primary limited and primary intense mating stage showed much lower amounts and could not be used for estimation.

Table 8. Concentration of 21 female specific compounds estimated per individual determined from secondary limited / intense mating extracts of female abdominal tip.

No	Compound	Concentration (ng)
68	α-Pinene*	0.04
69	β-Pinene*	0.07
70	p-Cymene	0.05
71	Limonene*	0.05
72	Geranylacetone	0.005
73	Tricosa-2,4-diene	0.03
74	Pentacosa-2,4-diene	0.03
75	Propyl acetate	1.3
76	1-Methylpropyl acetate	0.19
77	2-Methylpropyl acetate	0.42
78	3-Methylbutyric acid	0.18
79	2-Methylbutyric acid	0.18
80	Hexanoic acid	0.07
81	Phenylacetaldehyde	0.17
82	Benzoic acid	0.11
83	Octanoic acid	0.10
84	(*E*)-2-Decenal	0.36
85	Indole	0.84
86	(*E*)-2-Undecenal	0.15
87	(2*E*,4*E*)–2,4-Dodecadienal	0.15
88	4-Dodecanolide	0.02

*Absolute configuration not determined

All the listed compounds (Table 5-9) from female and male *P. interrupta* were investigated by using GC-MS and structures were confirmed by comparing their mass spectra with those of the standard ones from NIST mass spectral library (version 2.0, 23 July 2008). Supporting conclusions were drawn from the calculation of retention indices. Structure proposals for compounds **79** and **80** were done by elucidating the mass fragmentation pattern (section 5.8) and proven by synthesis (section 5.9).

5.7.4 Composition of extracts from primary limited and intense mating state of males

Table 9. Composition of male primary limited and intense mating states (A-Abdomen; TOA-Tip of the abdomen; WB-whole body; W&T- Wings and thorax; present (+); absent (-))

RI	Compounds	A	TOA	WB	W&T
936	Furanone	+	+	+	-
1105	Nonanal	+	+	+	+
1174	2-Ethylhexanoic acid	+	-	+	-
1206	Decanal	-	-	+	-
1674	Heptadec-1-ene	+	+	-	-
1862	Nonadec-1-ene	+	+	-	+
1928	Methyl hexadecanoate	-	+	-	-
2063	Henicos-1-ene	+	+	+	+
2171	Docos-1-ene	-	-	+	-
2112	Nonadecan-2-one	+	+	+	+
2270	Tricos-1-ene	+	+	+	+
2369	Tetracos-1-ene	+	+	-	-
2468	Pentacos-1-ene	+	+	-	+
2569	Hexacos-1-ene	+	+	-	+
2671	Heptacos-1-ene	+	+	+	+
2776	Octacos-1-ene	+	+	-	-
2826	Squalene	+	+	+	+
2871	Nonacos-1-ene	+	+	+	+
3075	Hentriacont-1-ene	+	+	+	-

5.7.5 Composition of extracts from secondary limited and intense mating state of males

Table 10. Composition of male secondary limited and intense mating states (A-Abdomen; TOA-Tip of the abdomen; WB-whole body; W&T- Wings and thorax; present (+); absent (-))

RI	Compounds	A	TOA	WB	W&T
894	Ethyl pentanoate	+	-	-	-
1105	Nonanal	+	+	+	+
1190	Ethyl octanoate	+	-	-	+
1391	Ethyl decanoate	+	+	-	+
1585	Dodecanoic acid	+	-	-	-

RI	Compounds	A	TOA	WB	W&T
1594	Ethyl dodecanoate	+	-	-	-
1674	Heptadec-1-ene	+	+	-	+
1724	Methyl tetradecanoate	+	-	-	-
1782	Tetradecanoic acid	+	+	-	+
1794	Ethyl tetradecanoate	+	-	-	+
1799	Hexadecanal	+	+	-	-
1862	Nonadec-1-ene	+	+	+	-
1928	Methyl hexadecanoate	+	+	-	+
1976	Ethyl hexadecenoate	+	-	+	-
1981	Hexadecanoic acid	-	-	-	+
1994	Ethyl hexadecanoate	+	+	+	-
2001	Octadecanal	-	-	+	-
2063	Henicos-1-ene	+	+	+	-
2112	Nonadecan-2-one	-	+	+	-
2179	Octadecanoic acid	-	+	-	+
2186	Ethyl octadecenoate	+	+	+	+
2190	Ethyl octadecanoate	+	+	-	-
2270	Tricos-1-ene	-	+	+	-
2369	Tetracos-1-ene	-	+	-	-
2468	Pentacos-1-ene	+	+	+	+
2569	Hexacos-1-ene	+	+	+	+
2671	Heptacos-1-ene	+	+	+	+
2776	Octacos-1-ene	+	+	-	-
2826	Squalene	+	-	+	-
2871	Nonacos-1-ene	+	+	+	+

5.8 Structure elucidation of tricosa-2,4-diene (79) and pentacosa-2,4-diene (80)

The mass fragmentation pattern of the two female specific compounds **79** and **80** (Figure 54) that were identified from all the body parts of only female beetles was almost similar with a difference of increase in molecular ion by 28 amu indicating the existence of an extended carbon chain by C_2H_4 unit in the latter. Compound **79** showed a molecular ion at *m/z* = 320 with a base peak at *m/z* = 68 followed by *m/z* = 81 as one of the major peak (Figure 54; top) which are the characteristic ions for a system with alternating double bonds.[135] Its structure was proposed as tricosa-2,4-diene (**79**)**.** Structure of another female specific compound **80** which showed its molecular ion at *m/z* = 348 (Figure 54; bottom) was predicted as pentacosa-2,4-diene (**80**).

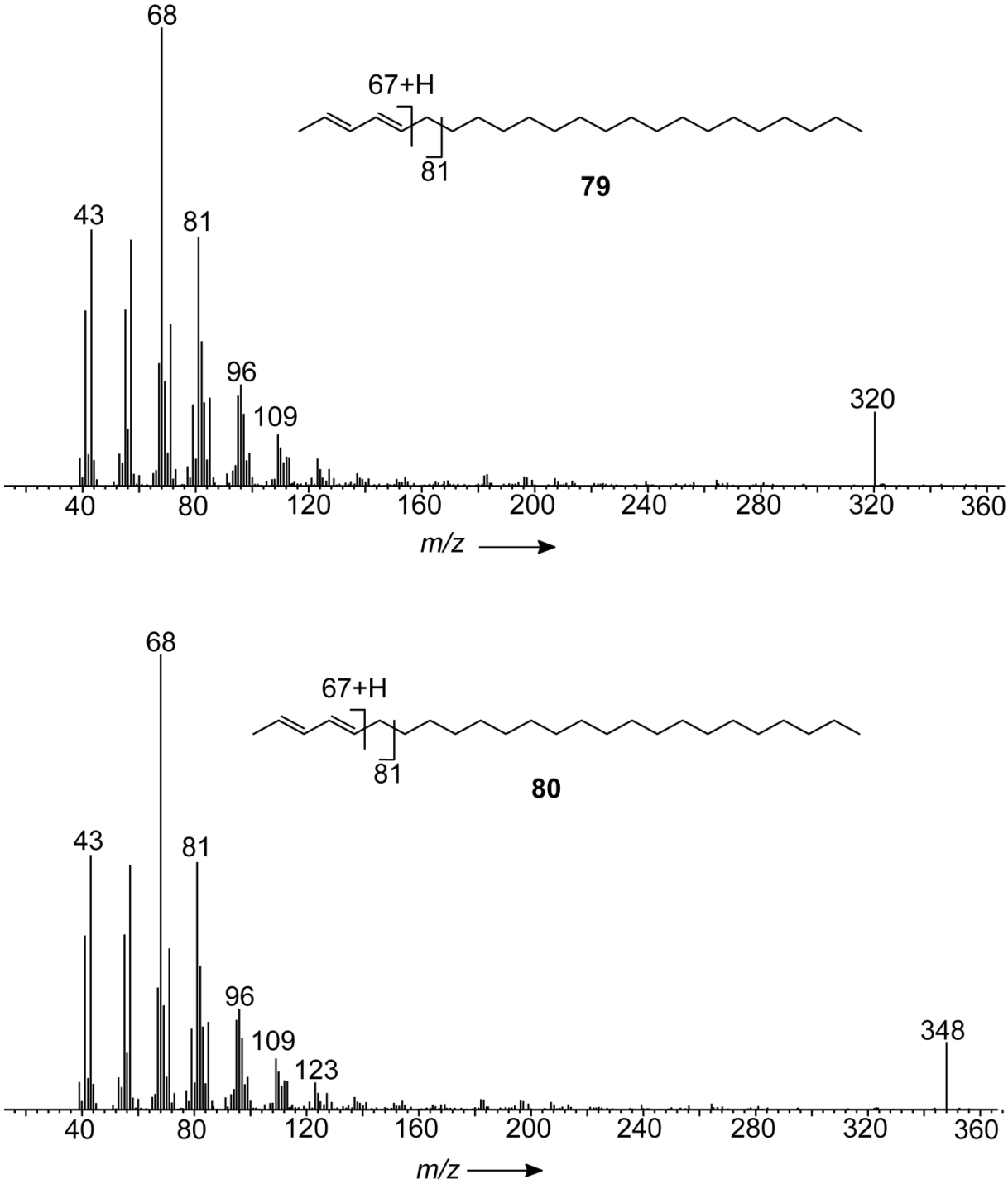


Figure 54. Mass spectra of female specific compounds **79** and **80** from *Pachnoda interrupta* Tricosa-2,4-diene (**79**) (top); Pentacosa-2,4- diene (**80**) (bottom).

5.9 Synthesis of two female specific compounds 79 and 80

5.9.1 Synthesis of tricosa-2,4-diene (**79**)

The compound **79** identified only in the female extracts was synthesized starting with nonadecanoic acid (**95**) which upon treatment with the Lewis-acid BF_3*$(Et)_2O$ in methanol[108] gave the corresponding methylester **96.** Reduction of **96** with LAH proceeded under dry conditions giving its respective alcohol **97** with a high yield of 98%.[136] Oxidation of **97** under Swern reaction conditions by using oxalyl chloride, DMSO and trimethyl amine as base furnished its aldehyde **98**.[137]

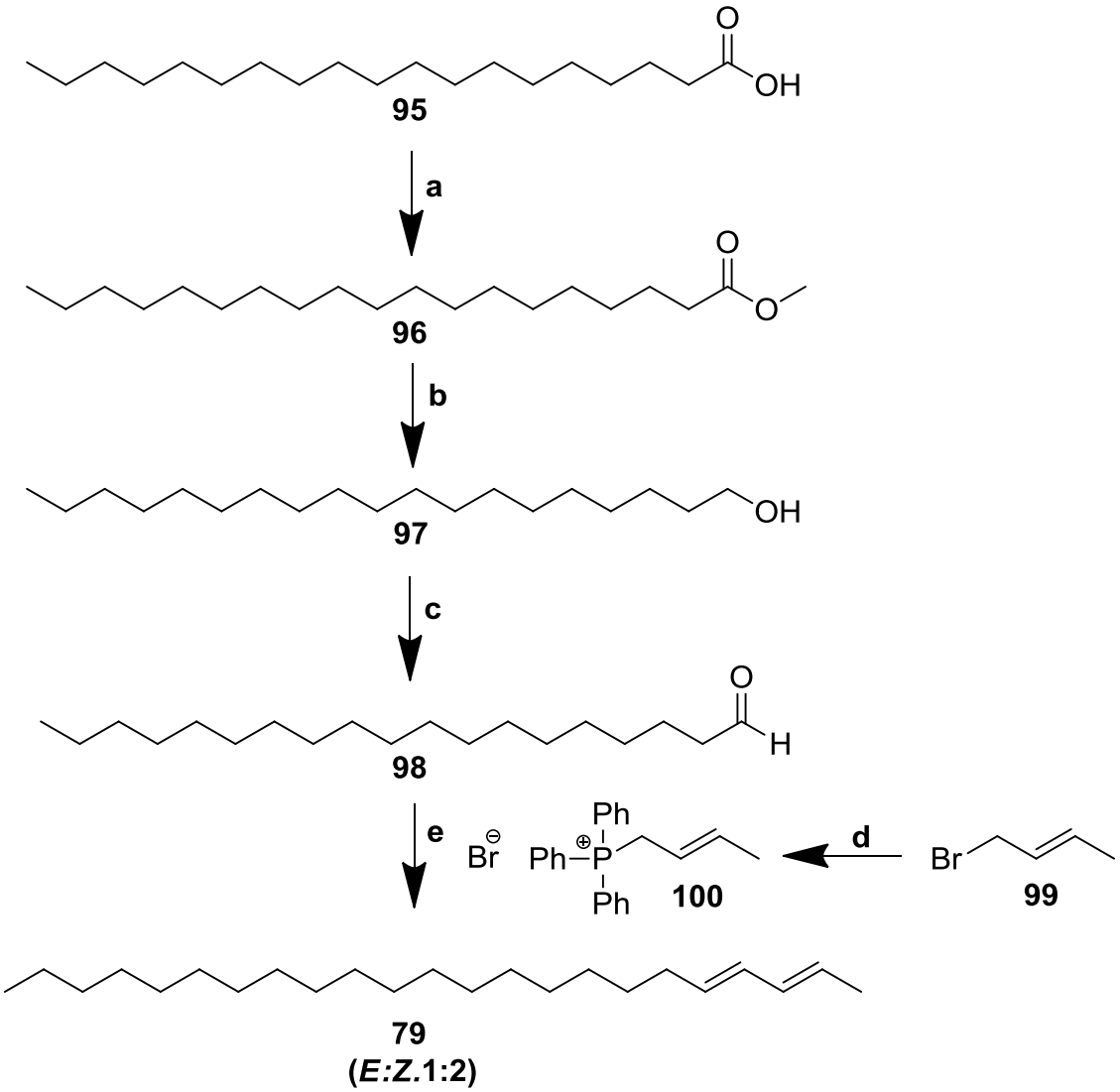
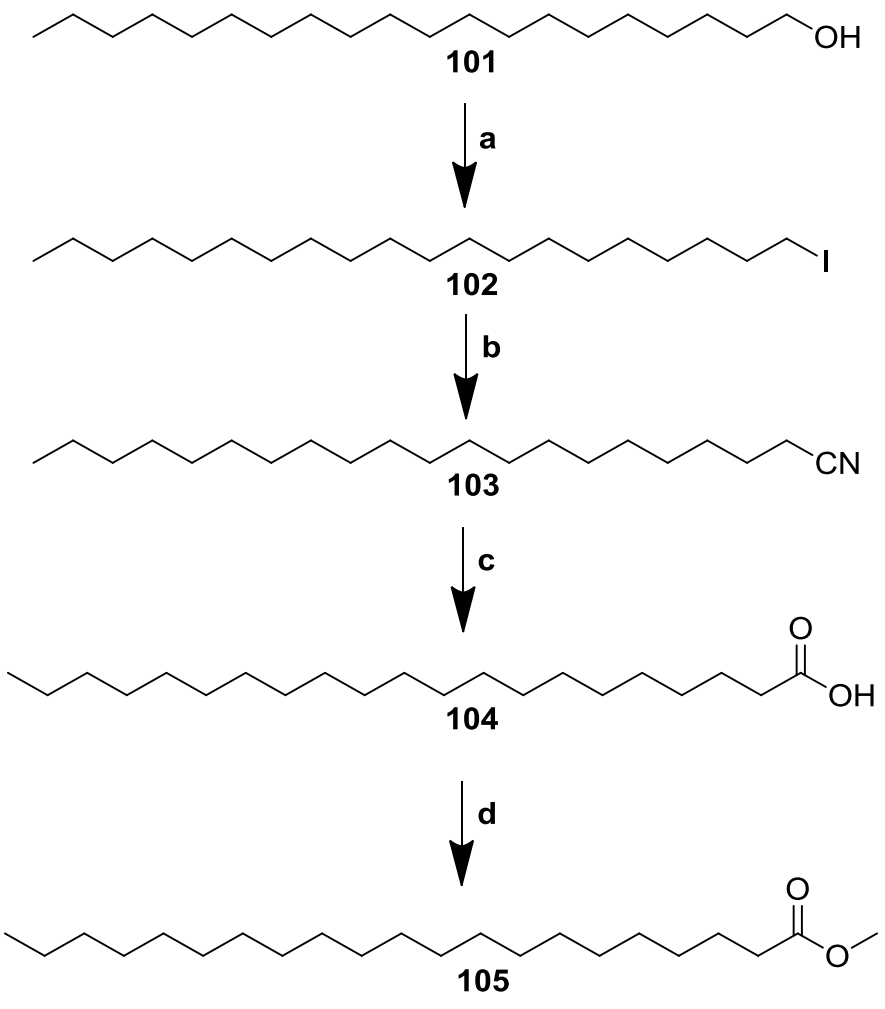


Figure 55. Synthesis of racemic tricosa-2,4-diene (**79**) **a**) $BF_3{\cdot}(Et)_2O$, MeOH, 0°C, 97% **b**) $LiAlH_4$, abs. ether, 0°C, 96% **c**) $(COCl)_2$, DMSO, $N(Et)_3$, 61% **d**) PPh_3, abs. toluene, 110°C, 48 h, 93% **e**) Li-HMDS, abs. DME, -78°C, 49%.

Wittig reaction of the aldehyde **98** using Li-HMDS,[138] and crystalline triphenyl phosphonium salt **100**, which was in turn obtained by the treatment[139] of trans-crotyl bromide (**99**) (85:15 *E*/*Z* mixture) with PPh_3 yielded the desired conjugated diene **79** as *E*/*Z* 2:1 (GC) product mixture. The stereochemistry of the double bond could not be assigned because the starting compound was a 85:15 *E*/*Z* mixture, and also only two peaks were obsereved in GC.

5.9.2 Synthesis of pentacosa-2,4-diene (**80**)

The approach used for the synthesis of target molecule **80** was almost similar to the one explained above (section 5.9.1) except for the chain elongation of the starting material 1-eicosanol (**101**) by one carbon unit. Halogenation of **101**, using triphenyl phosphine, imidazole and iodine,[140] followed by the transformation of **102** into its corresponding nitrile **103** by treatment with tetraethyl ammonium cyanide in DMSO ensured **103**.[141] Subsequent hydrolysis of **103** using NaOH in aqueous ethanol yielded the acid **104**.[142]



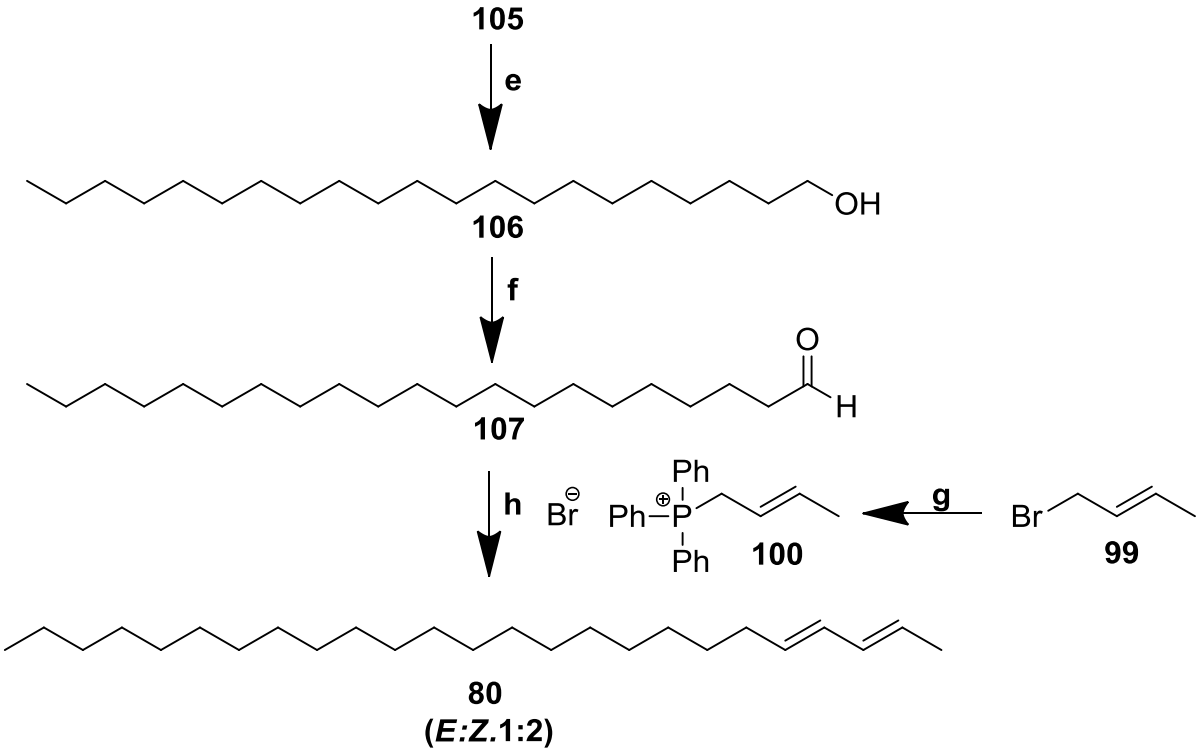


Figure 56. Synthesis of racemic pentacosa-2,4-diene (**80**) **a**) imidazole, PPh_3, I_2, 0°C, 41% **b**) DMSO, $(Et)_4NCN$, 100% **c**) NaOH, 60% Ethanol, 100°C, 24hrs, 95% **d**) $BF_3{\cdot}(Et)_2O$, MeOH, 0°C, 80% **e**) $LiAlH_4$, abs ether, 0°C, 100% **f**) $(COCl)_2$, DMSO, $N(Et)_3$, 69% **g**) PPh_3, abs.Toluene, 110°C, 48hrs, 93% **h**) Li-HMDS, abs.DME, -78°C, 42%.

Acid catalyzed esterification of **104** to **105,**[108] followed by LAH reduction of **105** provided molecule **106**.[136] Swern oxidation of **106** furnished **107**.[137] The final step of in the synthesis of pentacosa-2,4-diene (**80**) involved the formation of a carbon-carbon double bond between **107** and **100** via a Wittig reaction involving Li-HMDS.[138] The major isomers in synthetic **79** and **80** showed identical retention indeces when compared to natural compounds.

5.10 Field trapping experiments with female specific compounds

Field trapping experiments performed during the mating season of *P. interrupta* with live beetles as bait demonstrated the attraction and aggregation of males and females only to unmated females indicating the existence of a female released sex pheromone. In order to confirm the presumption, trapping experiments with the screened female specific compounds were performed in the field. Since the absolute configuration of the α-Pinene (**74**), β-Pinene (**75**) and limonene (**76**) in the natural extracts was not determined due to the low concentration, three isomers of pinene

and two enantiomers of limonene were included (Table 11). The peak corresponding to 2-methylpropyl acetate (**83**) was not clearly identified at the time of field testing hence, this compound was not included. Also the conjugated dienes **79** and **80** which were not synthesized at the time of field traps were also excluded from the trapping experiments.

All the field trap experiments were performed by using Japanese beetle traps that were suspended from wooden poles ca. 3 m above the ground. Investigations related to activity patterns of male and female adults during the mating season was carried out in unused farmland with sparse vegetation containing scattered *Acacia* spp. trees near the village of Embuay Bad in Ethiopia (09°48'N,40°00'E), 265 km northeast of Addis Abeba, 1206 m above sea level, during the month of July 2008. Traps baited with banana showed high attractivity which was previously demonstrated[134] and hence, for these experiments half banana was used as a bait which was mashed and placed in the collection chamber. Traps were placed throughout the site, with a minimum distance of 50 m between each trap.

Emission rates for dispensers were tested in a cylindrical wind tunnel (1 m long, 60 cm diameter, 0.25 m/s air flow, 25°C) that were replaced in the field each morning, just before the active period of adult *P. interrupta*. The release rates were calculated for the first 8 hours. For comparison, release rates of plant compound lures that had been previously shown attractivity in the field[134] was determined in this wind tunnel. A release rate of 0.5-1 mg/h during the 8 hours of measurement was observed. Thus the combination of dose and dispenser for the female compounds was chosen to ensure a similar emission rate.

The least volatile compounds were put on cotton rolls. For more volatile compounds, cotton rolls were pushed into 4 ml glass vials until the cotton was level with the rim of the opening of the vial (Table 11 "cotton roll in vial"), closed with a cap. A hole was made into the cap and the cotton roll was placed so that it was in direct contact with the cap when the cap was screwed tightly to the vial. The test compounds were applied to the cotton roll after placement in the vial.

Two variants were made of this dispenser, one for the most volatile compounds, where the chemical was applied towards the edge of the vial ("capped vial, distal application", Table 11). For compounds that were less volatile, compound was

applied just underneath the hole in the cap (Table 11 "capped vial, centered application"). All compounds were applied as neat solution, apart from solid indole (**91**) and benzoic acid (**88**) that were dissolved in tetrahydrofuran (99+% (GC), at a concentration of 33%.

Each compound was tested for its activity singly and as a mixture. In case of mixture all individual compounds were applied to separate dispensers, placed together in a round plastic container (diameter 68mm, height 30mm) and secured with a metal wire. For comparison, treatments with live unmated male and female *P. interrupta* in wire mesh cages as bait were also included. Beetles that were used as bait were excavated from aestivation sites near Rasa in May 2008.

Table 11. Treatment for field tests of female compounds. The numbers refer to Figure 50.

No	Compound	Dispenser	Dose
68	(+)-α-Pinene	capped vial, distal application	50mg
68	(-)-α-Pinene	capped vial, distal application	50mg
69	(1*S*)-(-)-β-Pinene	capped vial, distal application	50mg
70	p-Cymene	capped vial, centered application	100mg
71	(*S*)-(-)-Limonene	capped vial, centered application	100mg
71	(*R*)-(+)-Limonene	capped vial, centered application	100mg
72	Geranylacetone	cotton roll	100mg
75	Propyl acetate	capped vial, distal application	50mg
76	1-Methylpropyl acetate	capped vial, distal application	50mg
78	3-Methylbutyric acid	cotton roll in vial	50mg
79	2-Methylbutyric acid	cotton roll in vial	50mg
80	Hexanoic acid	cotton roll	100mg
81	Phenylacetaldehyde	cotton roll	100mg
82	Benzoic acid*	cotton roll	65mg
83	Octanoic acid	cotton roll	200mg
84	(*E*)-2-Decenal	cotton roll	100mg
85	Indole*	cotton roll	65mg
86	(*E*)-2-Undecenal	cotton roll	100mg
87	(2*E*,4*E*) – Dodecadienal	cotton roll	50mg
88	4-Dodecanolide	cotton roll	200mg
-	Mixture**	plastic container	as above
-	Unmated females	mesh cage	5 beetles
-	Males	mesh cage	5 beetles
-	Control- THF	cotton roll	200mg
-	Control empty	none	none

*200 mg of 33% solution in THF ** all single compounds (including isomers).

The results from the field experiments showed that the traps baited with phenylacetaldehyde (**87**) caught considerably more beetles than all other treatments (Figure 57), catching 1191 beetles per trap, followed by an average of 64 beetles per trap for hexanoic acid which was the second most attractive compound. The mixture of all compounds caught significantly less than empty control traps or control traps with a solvent blank. Sex ratios were male-biased for all treatments.

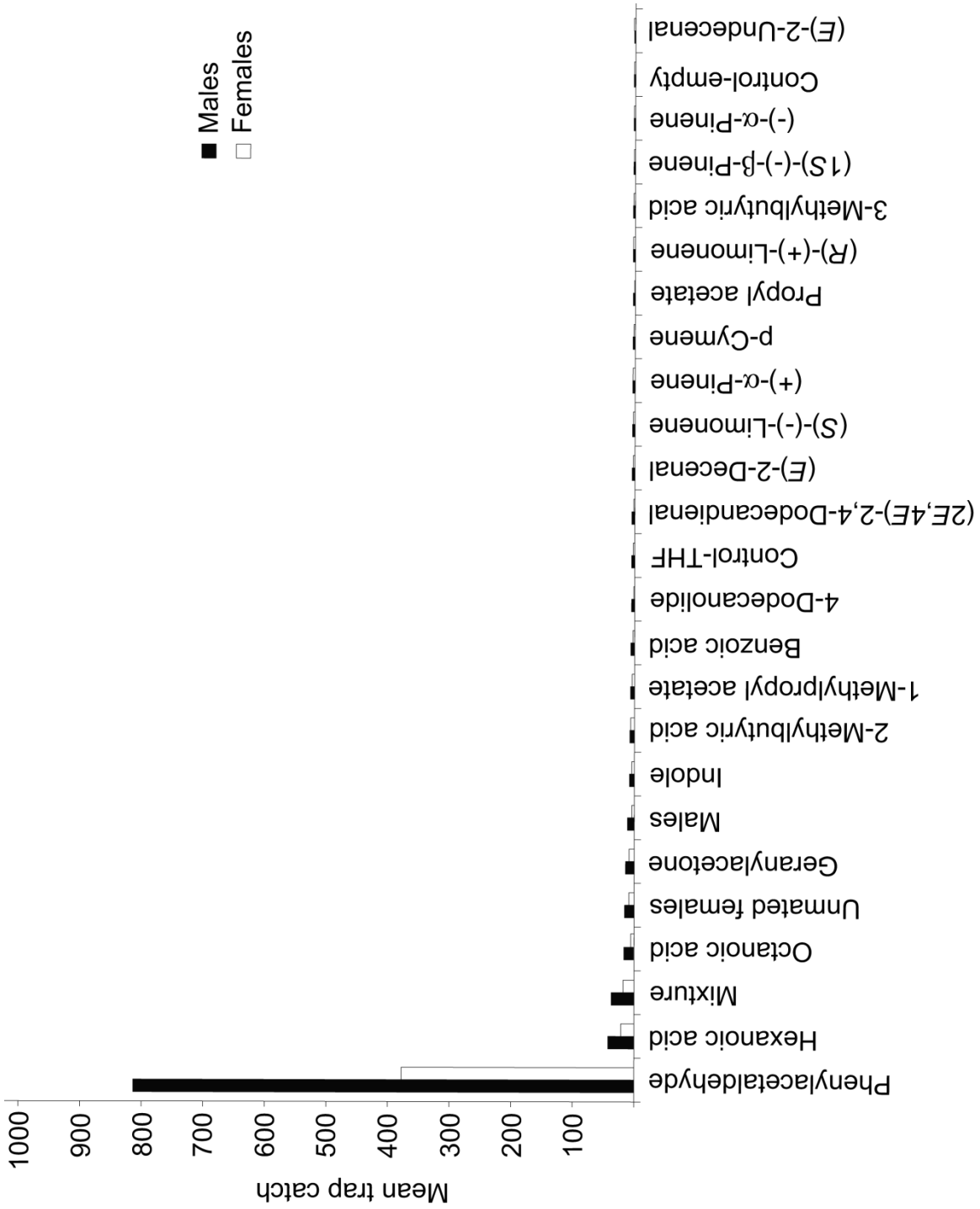


Figure 57. Mean trap catch of *P. interrupta*

The field experiments also showed that the traps containing single compounds as well as mixtures combined with mashed banana attracted significantly more beetles than the compounds alone, which might likely caused by an interaction between female pheromone signaling system and the host volatiles as evidenced by the strong aggregation of both sexes to traps.

The sorghum chafer showed tendencies of protandry in the mating season.[143] During the first eight days of the trapping period, the average trap catch was moderate (Maximum average daily catch was 29 beetles per trap) and there was a strong male bias in catch (>90%). From the day 9 onwards beetle activity increased, but the proportion of females remained low. Female activity drastically increased on day 14, making the sex ratio approximately even. With the onset of heavy rains from day 15 (mid of July), the beetles ceased flying and were not observed mating or feeding on hosts.

The mixture of all identified female specific compounds (excluding **79**,**80** and **83**) caught significantly less beetles than traps baited with phenylacetaldehyde (**87**) alone (Figure 57), indicating that the ratios of compounds tested did not match to those released by unmated females, or that compounds vital for attraction are missing from the blend. The low level of attraction to the mixture might also be due to the presence of one or more compounds in the mixture that might be inhibiting the attraction. In the field experiments performed, attraction to the negative control traps was very low, making it difficult to draw any conclusions regarding inhibition of attraction by any of the single compounds tested (Figure 57).

The extensive field trap experiments were performed by Dr. Jonas M. Bengtsson (Swedish University of Agricultural Sciences, Alnarp, Sweden), Dr. Yitbarek Wolde Hawariat (Swedish University of Agricultural Sciences, Alnarp, Sweden), and Merid Negash (Jena, Germany) who were co-workers in this project.

5.11 Conclusion

GC-MS investigations of extracts from different body parts of male and female beetle *P. interrupta* revealed the existence of 21 female specific compounds **74-94** (Figure 50-53) that were exclusively found in abdominal tip. Field experiments with these compounds (both singly and as mixture) showed that one of the female specific compounds, phenylacetaldehyde (**87**), caught significantly more beetles than any other trap (Figure 57).

Phenylacetaldehyde (**87**) is a common constituent of flower volatiles[144] which was also reported as a pheromone system in Australian *Polyrhachis* weaver ant.[145] The use of plant volatiles as pheromone components has been previously reported in several insects.[146-148] However, field experiments with phenylacetaldehyde (**87**) proved that it also attracts male beetles resulting in formation of aggregations containing both sexes (Figure 57). The experiments performed with the female specific compounds in combination with food source (banana) attracted more beetles (both the sexes) showing that aggregations might thus be influenced by the pheromone dose as well as the host volatiles. Similar pattern involving increased attraction of conspecifics in combinations to pheromones with volatiles from hosts has been studied in e.g. *Popillia japonica*[149] (Coleoptera:Scarabaeidae) and in the diamond black moth, *Plutella xylostella*[150] (Lepidoptera:Plutellidae). Insects feeding on plants may alter the volatile profile of their respective hosts by feeding damage or inoculation[151] or might induce the emission of volatiles that are attractive to conspecifics facilitating aggregations[152] and mating behavior.[153]

As *P. interrupta* form aggregations during the mating season simultaneous access to food and mates could also be an advantage for both male and female beetles[143] although several drawbacks are associated with aggregation, e.g. increased competition, predation, and pathogen pressure. However, the full evaluation of aggregation behaviors in *P. interrupta* would need further studies.

The location of pheromonal glands in *P. interrupta* is not known. Since, the GC-MS analysis involving the extracts from different parts of the body showed many female specific compounds in the extracts of the abdominal tip. It might be possible that pheromone-producing structures may be located in this abdominal tip, e.g. in a gland

in the abdomen tip, or in epithelial cells on the inner surface of the cuticle, as known in other scarab species.[154]

The attraction of both male and female beetles of *P. interrupta* to the possible pheromone compound phenylacetaldehyde (**87**) during the adult stage which is the damage causing stage of life, is promising for future applications. Trapping of pest species where adults are the damage causing stage have been proven efficient in controlling them.[155] Phenylacetaldehyde (**87**) could be combined with previously identified host-related attractants, such as methyl salicylate (**71**), eugenol (**72**),[134] and 2,3-butanediol (**73**),[133] for evaluation of synergistic effects, and to further improve the attractiveness of lures. Such lures could be powerful instruments for monitoring, and also for large-scale trapping of *P. interrupta*.

6. Pheromone biosynthesis in the winter moth *Operophtera brumata*

6.1 Life style of *Operophtera brumata*

The winter moth *O. brumata* (Lepidoptera:Geometridae) is a common forest pest that is native to Europe and an invasive species to Northern America. Over the decades it has become a serious defoliator of several deciduous plants that include oaks, maples, basswood, white elm, crabapple, apple, blueberry, and flowering plum. The females of *O. brumata* are small (8mm) and wingless, while the males are also small (Figure 58) with fringed appearance but including the wing span they appear larger (25mm).[156]

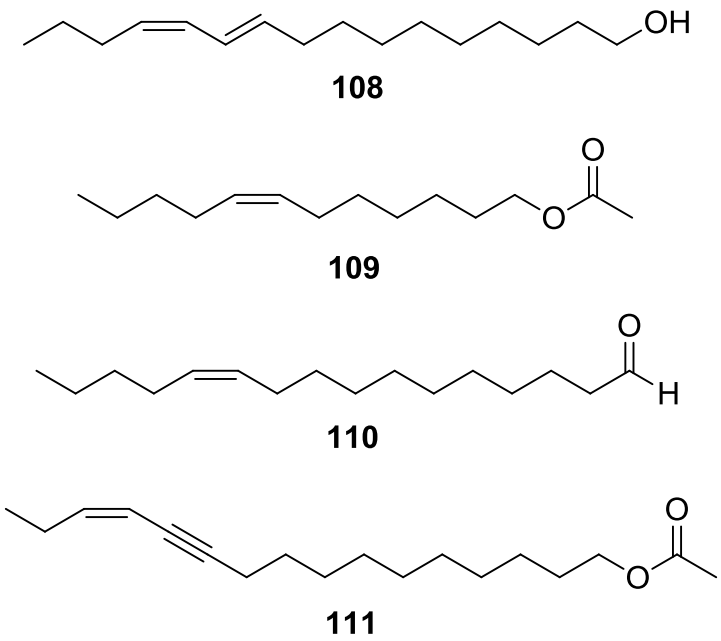
Figure 58. Picture showing *Operophtera brumata* (Left: Female, Right: Male) (Photo: Ralph Stewart)

Young larvae are tiny inchworms which tunnel into buds when they feed, especially preferring fruit trees. Older larvae feed on the expanding leaf clusters and are capable of creating defoliations in high populations. Such kind of extensive defoliation might lead to branch mortality or even some times tree mortality. The adult stage of the winter moth emerges from soil usually in late November and can be active until January. Since the females are wingless they cannot fly, in turn they crawl to the top of the trees and wait for males or are carried onto other trees with the help of silken threads released by them along the wind, a process called ballooning.

After mating the female deposits egg clusters on tree trunks, branches, or in bark crevices which hatch in spring seasons when the temperature is around 12°C. After hatching the young larvae also move by the process of ballooning and feed voraciously until mid June, where upon they migrate into the soil for pupation. The pupae stage is passed in soil until they emerge as adults in winter. The adults are hard to control as they are huge in number, hence better to attack them at the caterpillar or pupal stage. Controll was successfully attempted by introduction of parasitoids or by predation employing natural predators.[157]

6.2 Introduction on Lepidopteran sex pheromones

Lepidopteran sex pheromones occurring in different moth species are diverse and are classified into two groups, called type I and type II pheromones. Most of the type I moth pheromones feature an even numbered carbon skeleton (dominated by $C_{10\text{-}18}$) containing a polar functional group at the terminal end, which might be an alcohol, acetate, or aldehyde. They are also characterized by 0-3 double bonds which orient in either (*E*)- or (*Z*)-configuration and often are conjugated.[158]



108 **109** **110** **111**

Figure 59. Common type I moth pheromones. **108** *Bombyx mori*; **109** *Trichoplusia ni;* **110** *Heliothis virescens*; **111** *Thaumetopoea pityocampa.*

The structures illustrated in Figure 59 include (10*E*,12*Z*)-10,12-hexadecadien-1-ol (**108**), the first characterized pheromone of the silkworm moth *B. mori*.[159] Furthermore (*Z*)-7-dodecenyl acetate (**109**) initially identified as pheromone in *T. ni* that also serves as a pheromone system for 25 other species,[160] (*Z*)-11-hexadecenal (**110**) primarily identified as a pheromone of *H. virescens* but currently known to be present in 40 other species are depicted.[158]

In type I pheromones the straight chain with even numbered carbon skeleton is predominately formed as they are derived de novo via saturated fatty acids like palmitic acid (C-16) and stearic acid (C-18) which are desaturated via a fatty acyl intermediate in the pheromonal gland.[158]

Second class or type II pheromones in moth species are generally polyunsaturated hydrocarbons and corresponding epoxides (C_{17-23}), of which the latter might be a result of double bond oxidation.[161] Type II pheromones are a characteristic feature of macro-lepidopteran families like Geometridae, Acrtiidae, Noctuidae, and Lymantriidae. The additional double bond increases the diversity of type II pheromones. Figure 60 illustrates a few examples of this type. *Erannis bajaria* emits (3*Z*,6*Z*,9*Z*)-3,6,9-octadecatriene (**112**) and (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene (**113**) as pheromone blend,[162] the (6*Z*,9*S*,10*R*)-9,10-epoxyoctadecene (**114**) and (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxyoctadecadiene (**115**) are the sex pheromone components of the mulberry looper *Hemerophila atrilineata*.[163]

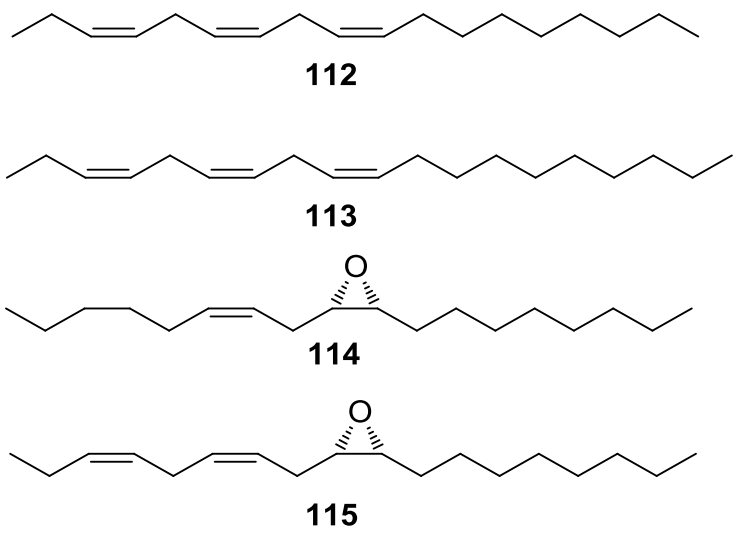
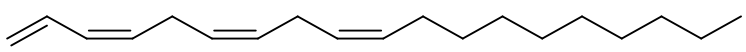


Figure 60. Common type II moth pheromones. **112** and **113** pheromone components of *Erannis bajaria*; **114** and **115** pheromone components of *Hemerophila atrilineata*

The biosynthesis of type II pheromones is derived from linoleic acid or linolenic acid which results in the formation of an odd or even numbered carbon skeleton with (*Z*)-arrangement of the double bonds. Subsequent regio- and stereoselective oxidation of one or two of the double bonds produce the corresponding epoxides.[161]

6.3 Pheromone of the winter moth *Operophtera brumata*

Previous studies revealed that *O.brumata* utilizes a pheromone system that operates at low temperatures between 4°C to 15°C. Using behavioral assays and electroantennographic (EAG) studies on abdominal tip of the female, the sex pheromone of the winter moth was identified as (3*Z*,6*Z*,9*Z*)-1,3,6,9-nonadecatetraene (**116**). Field trials with synthetic compound of **116** in a sustained flight tunnel attracted high number of males. The response of the males was optimal between 7°C to 15°C with a significant loss of activity below 4°C and above 17°C. The wingless females of the winter moth depend on this sex pheromone for communication and mating that works at low temperatures (around 16°C) as their pupal diapause is broken only at the later half of the fall season. It also shows that the males have adapted their mating communication system that is active for an extended period of time in an seasonally unfavorable conditions. The same pheromone molecule **116** also attracts two other geometrid species, *Operophtera bruceata* and *Operophtera occidentalis*.[156]



116

Figure 61. Sex pheromone of the winter moth *Operophtera brumata*

6.4 Biosynthetic considerations of the sex pheromone in *Operophtera brumata*

The possible biosynthesis of odd numbered tetraene molecules characterized with multiple double bonds separated by methylene bridges with the carbon chain ranging from C17-C23 can be originated from diet-derived linoleic acid or linolenic acids.

Similarly the biosynthesis of sex pheromone in *O. brumata* can be predicted (Figure 62) starting with α-linolenic acid (**117**) which undergoes a chain elongation by two carbon units giving raise to molecule **118**, followed by an enzymatically driven additional desaturation furnishing tetraene **119**. The target pheromone molecule **116** arises from reductive decarboxylation of even-numbered acyl precursor.[161]

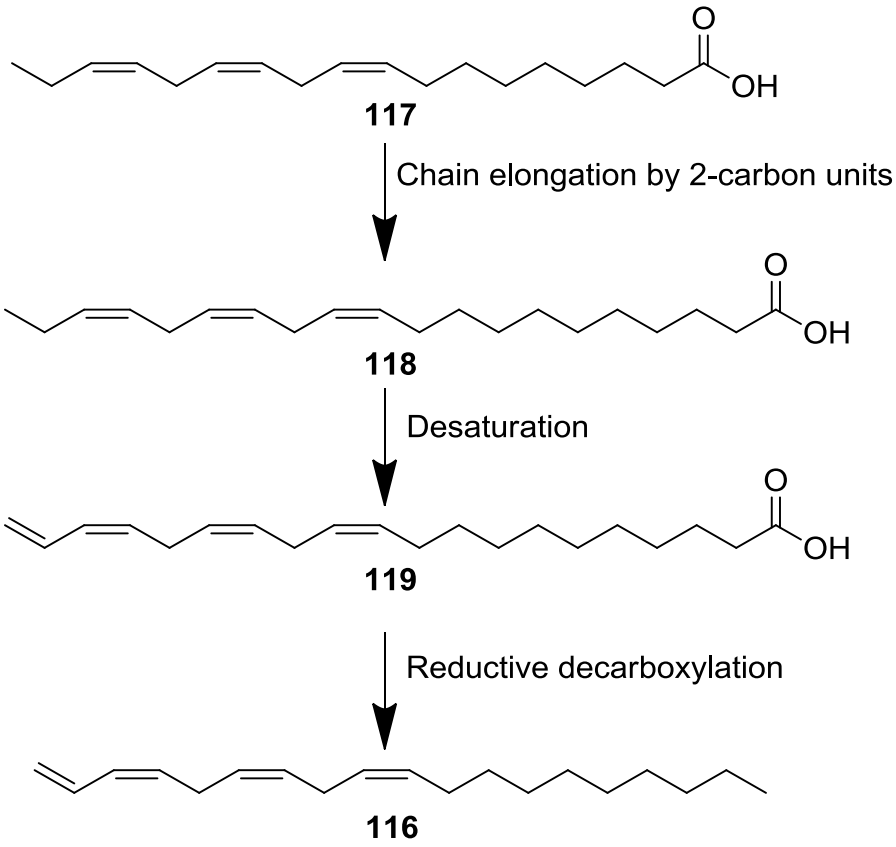


Figure 62. Possible biosynthesis of (3*Z*,6*Z*,9*Z*)-1,3,6,9-nonadecatetraene (**116**).

The above illustrated sequential mechanism for the biosynthesis of tetraene molecule **116** was also proven by the investigations which involved in the incorporation of labeled α-linolenic acid into the major pheromone component (3*Z*,6*Z*,9*Z*)-1,3,6,9-nonadecatetraene (**116**). Detection of fatty acyl intermediates **118** and **119** support the proposed biosynthetic pathway.[164]

6.5 Retrosynthetic plan for labeled pheromone precursors

To trace out the biosynthesis of the sex pheromone **116** via deuterium labeled precursors, the synthesis of (11*Z*,14*Z*,17*Z*)-methyl 2,2,3,3-tetradeuteroicosa-11,14,17,19-tetraenoate (**120**) was attempted. For this purpose a retrosynthetic plan (Figure 63) was designed cleaving the complex target molecule **120** into much

simpler and shorter molecules as illustrated (Figure 63). Acrolein (**121**) is a commercially available molecule whereas the other two precursors **122** and **123** can be achieved by performing multiple synthetic reactions that are discussed in detail in the following section 6.6.

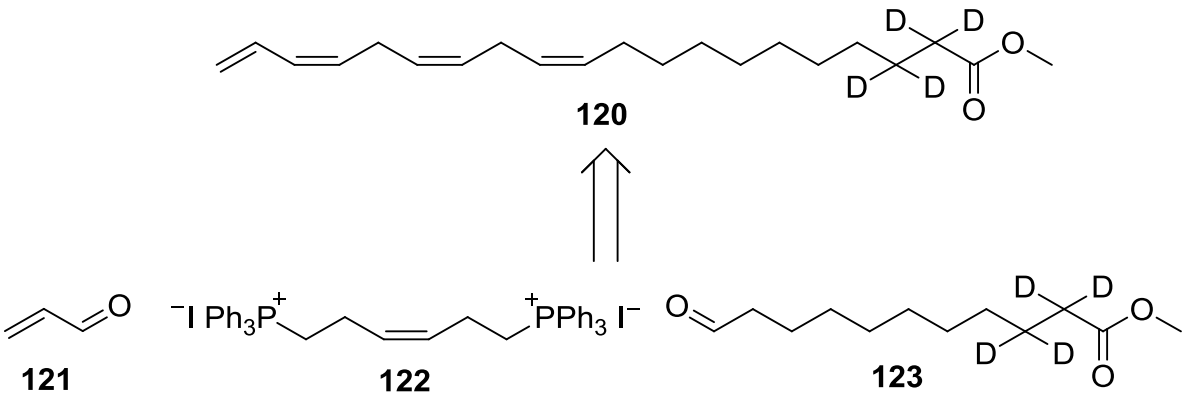


Figure 63. Retrosynthesis of labeled pheromone precursor

6.6 Synthesis of labeled pheromone precursors

6.6.1 Synthesis of the bifunctional Wittig salt 122

One of the precursor molecules **122** required for the synthesis of deuterium labeled tetraenoic acid derivative **120** was synthesized starting with an epoxidation of 1,4-cyclohexadiene (**124**) generating the corresponding epoxide **125** almost quantitatively.[165]

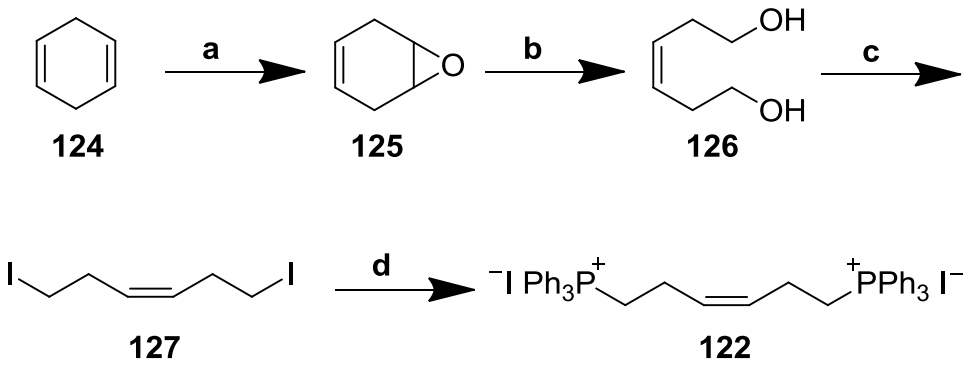


Figure 64. Synthesis of symmetric bifunctional Wittig salt **122** **a**) *m*-CPBA, K_2HPO_4, 0°C, 97%. **b**) 10% H_2SO_4, $NaIO_4$, $NaBH_4$, H_2O, THF, 54%, **c**) PPh_3, imidazole, I_2, 99% **d**) PPh_3, 52%.

A subsequent one-pot oxidative ring cleavage, followed by reduction of intermediate acyclic dial furnished (*Z*)-hex-3-ene-1,6-diol (**126**).[166] Halogenation of **126**, using triphenylphosphine, imidazole and iodine allowed its conversion to (*Z*)-hex-3-ene-1,6-diiodide (**127**) which was transformed into the crystalline symmetric bifunctional Wittig salt **122** (*Z*/*E* ≥ 95:5 determined by ^{13}C NMR spectroscopy) by treatment with triphenylphosphine.[167]

6.6.2 Synthesis of methyl 2,2,3,3,-tetradeutero-11-oxoundecanoate (123)

Synthesis of another target precursor **123** needed for the synthesis of isotopic labeled tetraene fatty acid derivative **120** was achieved starting with the esterification of 10-undecenoic acid (**128**) into its corresponding methyl ester **129**,[108] which was

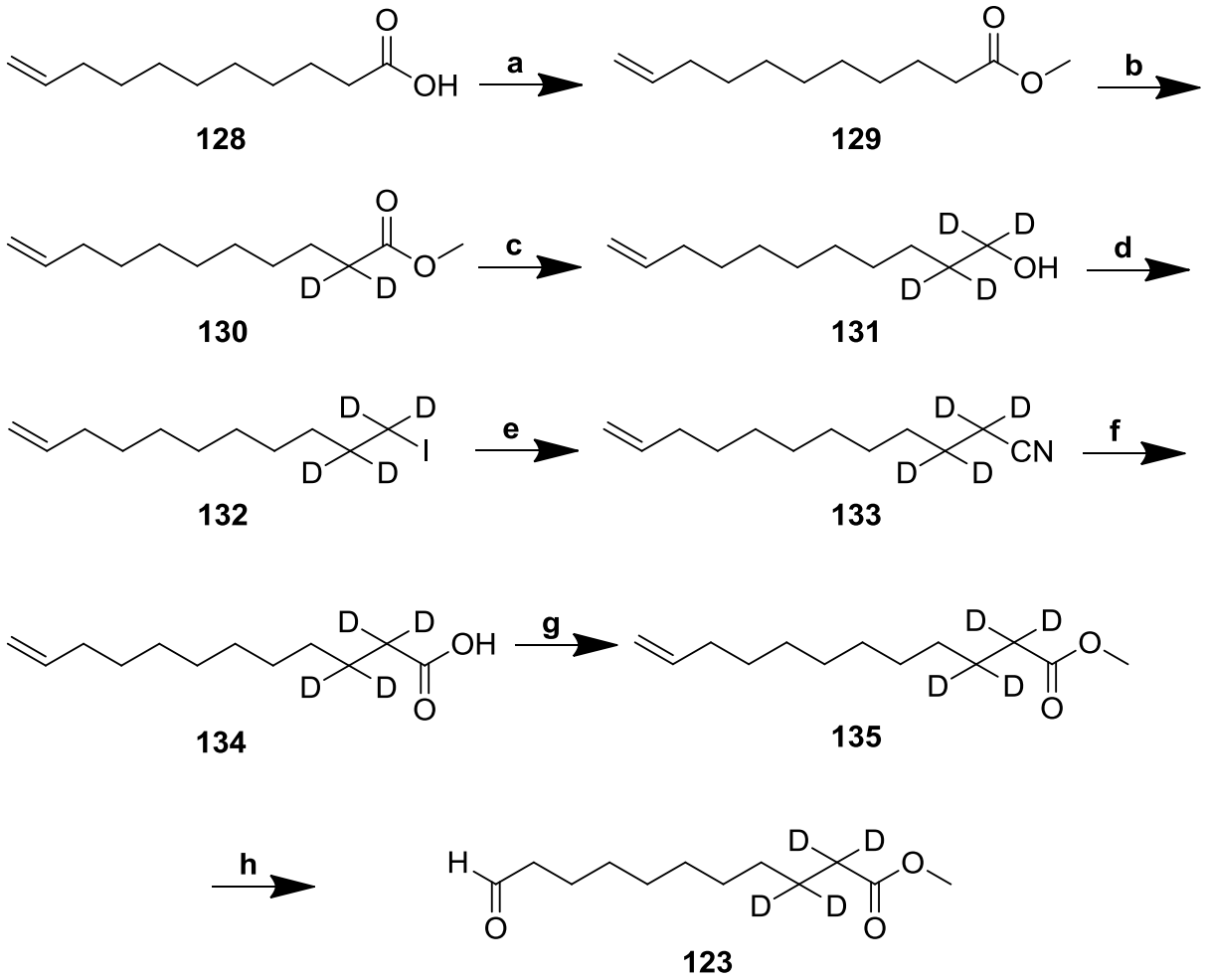


Figure 65. Synthesis of methyl 2,2,3,3,-tetradeutero-11-oxoundecanoate (**123**) **a**) $BF_3{\cdot}(Et)_2O$, MeOH, 0°C, 94%. **b**) Na, MeOD, 6 h Reflux, 23%, **c**) $LiAlD_4$, abs. ether, reflux, 98% **d**) imidazole, PPh_3, I_2, 0°C, 76% **e**) DMSO, $(Et)_4NCN$, 100% **f**) NaOD, 60% EtOD, 24h reflux, 58% **g**) EDC, MeOH, DMAP, 80%. **h**) $RuCl_3$, $NaIO_4$, 81%.

treated with an excess of D_1-methanol and sodium methoxide allowing hydrogen-deuterium exchange at the α-position yielding compound **130**.[168] Reduction of **130** with lithium aluminium deuteride provided the tetradeuterated alcohol **131**.[169] The corresponding Iodide **132** was obtained under Mitsunobu conditions,[170] which upon nucleophilic substitution with tetraethyl ammonium cyanide furnished the nitrile **133**.[141] Subsequent hydrolysis of **133** produced the acid **134**,[142] which upon treatment with EDC and methanol gave the corresponding methyl ester **135**.[79] Oxidative cleavage of the terminal double bond in the compound **135** with ruthenium chloride and sodium periodate gave the precursor molecule methyl 2,2,3,3,-tetradeutero-11-oxoundecanoate (**123**).[171]

6.6.3 One-pot double Wittig approach yielding labeled pheromone precursor

A novel one-pot double Wittig procedure was employed to generate the *cis* methylene-bridged labeled tetraene.[167] The reaction (Figure 66) involved the generation of a symmetrical bis(ylide) **136** from double Wittig salt **122**. This ylide was treated with the aldehyde **123** at -78°C, followed by slow warming of the reaction mixture to 0°C. After re-cooling acrolein (**121**) was added. This reaction allows an easy entry into homo-conjugated double bond systems, but is known for its low yields. Only trace amounts of the target compound **120** were detected.

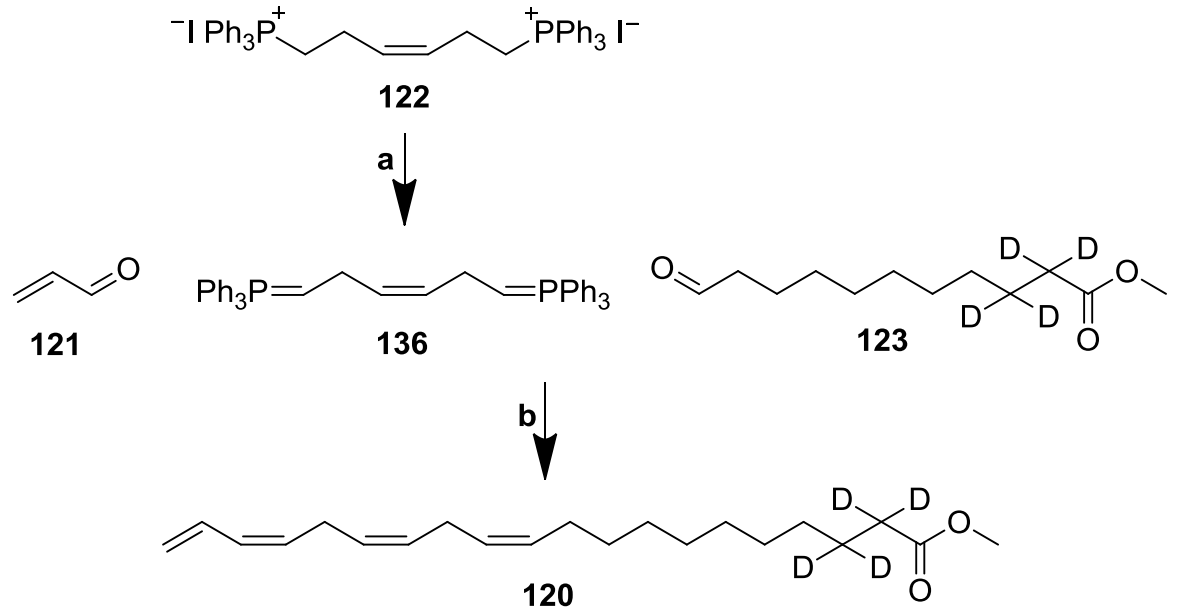


Figure 66. Synthesis of (11*Z*,14*Z*,17*Z*)-methyl 2,2,3,3-tetradeuteroicosa-11,14,17,19-tetraenoate (**120**) **a**) Li-HMDS, THF, -78°C **b**) DIBAL-H, abs.ether, -78°C, ≤1%.

The reactions in the synthesis of the labeled pheromone precursor compound **120** were complicated. Initially, formation of the bis(ylide) **136** was only possible after several optimizations and under controlled conditions. Reaction procedure involved monodeprotonation of the double-Wittig salt (with K/Na/Li-HMDS) and olefination, followed by a second deprotonation/olefination sequence that did not lead to the desired product formation. The desired cross-coupled product **120** could only be obtained in a mixture containing a major amount of the homo-coupled product when the aldehyde **123** was generated in situ through a slow decomposition of an aluminium alkoxide complex. The resulting cold reaction mixture obtained from the reduction of DIBAL-H was transferred to the ylide solution.

Incorporation experiments performed with the obtained impure product mixture of **120** were unsuccessful making the proposed desaturation of the terminal double bond in the biosynthesis (Figure 62) ambiguous. Furthermore, the synthesis of labeled pheromone precursors that can be used for incorporation studies before the desaturation step occurs was attempted as discussed in the following section 6.6.4.

6.6.4 Synthesis of methyl-(11*Z*,14*Z*,17*Z*) 3,3,4,4-tetradeuteroicosa-11,14,17-trienoate (142)

To establish the proposed sequential biosynthetic scheme (Figure 62), in which the desaturation is a key step, efforts were made to synthesize labeled pheromone precursors without the terminal double bond (Figure 67). The synthesis started with the conversion of α-linolenic acid (**137**) into the corresponding methyl ester **138**[108] which was treated with sodium methanolate and D_1-methanol to incorporate deuterium in a proton exchange reaction at the α-position yielding **139**.[168] Lithium aluminium deuteride reduction of **139** gave the respective tetradeuterated alcohol **140**,[169] which was subsequently converted into its relevant iodide **141** by reaction with triphenylphosphine, imidazole, and iodine.[170]

Deprotonation of methyl acetate at the α-position with LDA at deep temperatures generated the corresponding enolate which was captured ultimately by the electrophile **141** in a S_N2 reaction furnishing the target pheromone precursor methyl-(11*Z*,14*Z*,17*Z*) 3,3,4,4-tetradeuteroicosa-11,14,17-trienoate (**142**) in pure form, but with low yield (12%).[73]

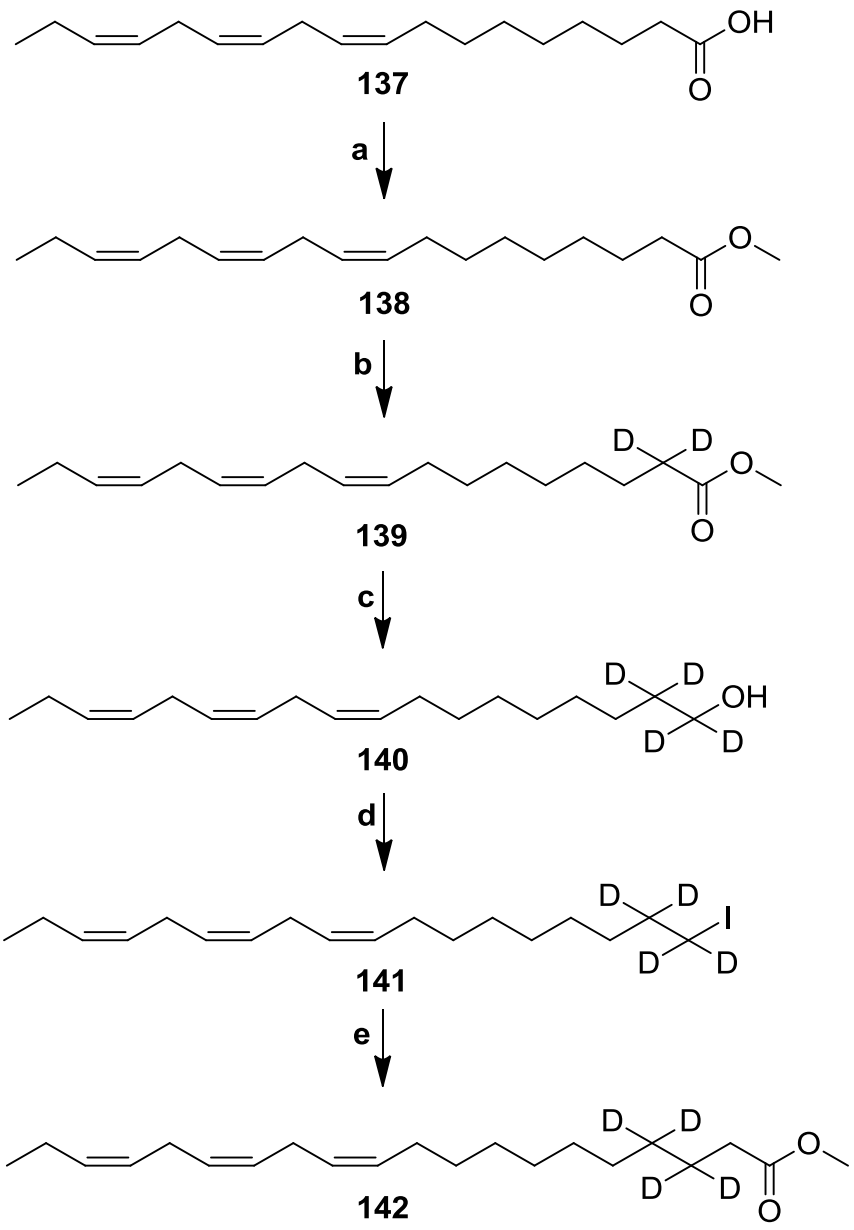


Figure 67. Synthesis of methyl-(11*Z*,14*Z*,17*Z*) 3,3,4,4-tetradeuteroicosa-11,14,17-trienoate (**142**) **a**) $BF_3{\cdot}(Et)_2O$, MeOH, 0°C, 98%. **b**) Na, MeOD, 4h reflux, 71%, **c**) $LiAlD_4$, abs. ether, reflux, 99% **d**) imidazole, PPh_3, I_2, 0°C, 84% **e**) LDA, -78°C, methyl acetate, 12%.

6.6.5 Synthesis of (11*Z*,14*Z*,17*Z*)-2,2,3,3,4,4-hexadeuteroicosa-11,14,17-trienoic acid (144)

A parallel synthesis was performed aiming at the deuterated acid **144** which was progressed by the deprotonation of acetonitrile using *n*-BuLi, followed by coupling with the cation generated from the analogous iodide **141** provided the alkyl cyanide **143**.[172] Hydrolysis of **143** with an excess of sodium deuteroxide and 60% D_1-ethanol (prepared from D_2O) introduced further more deuterium labelings at the α-position, affording the desired target molecule (11*Z*,14*Z*,17*Z*)-2,2,3,3,4,4-hexadeuteroicosa-11,14,17-trienoic acid **(144**) in reasonably good yield.[142]

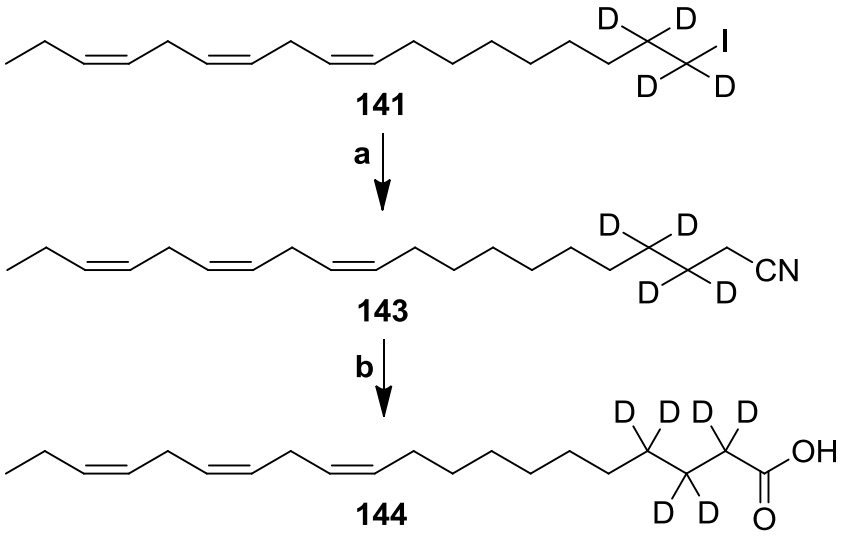


Figure 68. Synthesis of (11*Z*,14*Z*,17*Z*)-2,2,3,3,4,4-hexadeuteroicosa-11,14,17-trienoic acid (**144**) **a**) THF, *n*-BuLi, CH_3CN, -78°C, 30%. **b**) NaOD, 60% EtOD, 24h reflux, 45%.

Incorporation experiments to trace out the putative pheromone biosynthetic pathway (Figure 62) in the winter moth *O. brumata* with the isotopic labeled pheromone precursor compounds **142** and **144** synthesized in pure form is currently under investigation in the research group of Christer Löfstedt at Lund university, Sweden.

6.7 Conclusion

The Lepidopteran winter moth *Operophtera brumata* belonging to the family Geometridae uses (3*Z*,6*Z*,9*Z*)-1,3,6,9-nonadecatetraene (**116**) as sex pheromone to attract conspecifics.[156] This molecule belonging to the type II class of pheromones with conjugated double bonds and methylene bridges follows a different biosynthetic pathway when compared to type I Lepidopteran sex pheromones (Section 6.2). It originates from the diet derived α-linolenic acid rather than being synthesized de novo. The existence of the terminal double bond (at C-1 carbon atom) makes the pheromone molecule much more diversified along with extending the appropriately assumed biosynthetic proposal by an additional desaturation step (Section 6.4).[161]

The incorporation experiments performed with labeled (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoic acid detected the fatty acyl intermediates (11*Z*,14*Z*,17*Z*)-icosa-11,14,17-trienoic acid and (11*Z*,14*Z*,17*Z*)-icosa-11,14,17,19-tetraenoic acid indicating that the additional double bond is introduced before the final decarboxylation step.[164]

To prove the putative biosynthetic pathway that follows chain elongation (by C-2 carbon units), desaturation, and reductive decarboxylation (Figure 62) labeled pheromone precursors methyl-(11*Z*,14*Z*,17*Z*) 2,2,3,3-tetradeuteroicosa-11,14,17,19-tetraenoate (**120**), methyl-(11*Z*,14*Z*,17*Z*) 3,3,4,4-tetradeuteroicosa-11,14,17-trienoate (**142**) and (11*Z*,14*Z*,17*Z*)-2,2,3,3,4,4-hexadeuteroicosa-11,14,17-trienoic acid (**144**) were synthesized employing conveniently adaptive synthetic routes (Figure 64-68). Incorporation experiments with compound **120** were not successful whereas investigations with the other two labeled pheromone precursors **142** and **144** are currently under progress.

7. Summary

The sex pheromone of the wasp spider *Argiope bruennichi* which is widespread over Mediterranean area and Temperate zone was analyzed using the dynamic headspace technique. The web of the spider along with the volatiles released were extracted and analysed by GC-MS showing two female-specific compounds **13** which existed as a diastereomeric mixture in web and headspace extracts, and **14** which occurred only in female webs. Mass spectral interpretations (Section 3.11) along with comparison of retention indices established the structure of **14** as 3-octanoyloxy-γ-butyrolactone (**14**), whereas further derivatizations with MSTFA, followed by synthesis (Section 3.12) proved the structure of **13** as trimethyl methylcitrate (**13**) containing two stereogenic centers. The configuration of the female specific compound **13** released by the spider was assigned as 2*R*,3*S* (major isomer) and 2*S*,3*S* (minor isomer) by chiral resolution by gas chromatography (Section 3.13). The synthetic compound **13** proved its activity attracting males, for the first time in spiders in the field, confirming trimethyl methylcitrate (**13**) as the sex pheromone of the spider *A. bruennichi* (Section 3.14). The function of the other female specific spider compound, 3-octanoyloxy-γ-butyrolactone (**14**) that has been identified needs to be established.

Investigation of cuticular extracts from the spider *Argyrodes elevatus* revealed the presence of unusual esters not known from spiders before. GC-analysis of the cuticular extracts from the female spiders showed four major peaks, whereas the male spiders showed only a single major peak (Section 4.3). The mass spectra were analyzed and structural proposals were made. The unknown esters **47**-**51** (Figure 39) proved to be methyl-branched long-chain compounds with an internal location of the ester functional group. Elucidation of the functional groups as well as the position of the methyl branches were performed by analysis of mass spectra, use of gas chromatographic retention indices, microderivatizations, and non stereoselective synthesis.[32,33] Employing efficient enantioselective methods (Section 4.4 and 4.5), three isomers out of 32 possible stereoisomers have been synthesized. More enantioselective synthesis has to be performed to assign the stereochemistry for the natural compounds, and as well to study the stereochemistry-activity relationship, and existing sexual dimorphism that might point to an additional role of the lipids

beyond the usual water regulation e.g. behavioral activity in an so far unknown context.

The beetle *Pachnoda interrupta* (sorghum chafer) which is a serious pest on *Sorghum bicolor* in the Northern part of Ethiopia was collected and the extracts from all body parts were analyzed using GC-MS. The analysis showed 21 female specific compounds **74-94** that were exclusively found in the abdominal tip (Figure 52). Field testing with these compounds (with a an exception of **79**, **80** and **83**), both singly and in a mixture, in search of behaviorally active pheromone components, revealed the attraction of males to one of the female specific compound namely phenylacetaldehyde (**87**), that caught significantly more beetles than any other trap (Figure 57). It also caught females, causing aggregations indicating that it might be a possible pheromone compound that can prove helpful in future application of trapping the pest species. Furthermore, traps baited with a mixture of all the female specific compounds attracted significantly less beetles than single compounds alone, which indicates that one or more compounds are present that inhibit the attraction. The activity of the conjugated dienes **79** and **80** that were synthesized (Section 5.9) needs to be confirmed.

The biosynthesis of the sex pheromone (3*Z*,6*Z*,9*Z*)-1,3,6,9-nonadecatetraene (**116**) in the geometrid moth *Operophtera brumata* was proposed to follow the sequence of chain elongation of α-linolenic acid, Δ^{19} desaturation, and reductive decarboxylation (Figure 62). In order to resolve the biosynthetic pathway of this pheromone released, isotopic labeled polyunsaturated fatty acid and fatty acid methyl esters **120**, **142**, and **144** were synthesized (Section 6.6). Incorporation experiments with the labeled pheromone precursors are currently under investigation.

8. Experimental procedures

8.1 General methods

Chemicals

Commercially available starting materials were purchased from Sigma-Aldrich Chemie GmbH (Germany), Fluka Chemie GmbH (Switzerland), Merck (Germany), Acros Organics (Belgium), TCI Organic Chemicals (Belgium), Stream Chemicals (Germany), Janssen Chimica (Belgium), and Riedel de Haën AG (Switzerland) that were used without further purification except if otherwise stated. Technical solvents were distilled before use. For the reactions performed with dry solvents, THF was distilled over sodium and potassium with benzophenone, dichloromethane over calcium hydride, diethylether over $LiAlH_4$, ethanol and methanol over magnesium.

Reaction conditions

All reactions involving water-sensitive chemicals were carried out in overnight oven dried (100°C) clean glass equipment which was further dried with a heat gun before the start of the reaction under nitrogen atmosphere and magnetic stirring.

Thin layer chromatography and column chromatography

TLC was performed on 0.2 mm pre-coated plastic sheets of Polygram® SIL G/UV_{254} plates purchased from Macherey-Nagel. Detection of compounds was performed by immersion in 10% ethanolic solution of phosphomolybdic acid, or in detecting mixture containing 5g potassium permanganate, 20g K_2CO_3, and 5ml 1N NaOH in 300ml water, followed by gentle heating with a heat gun. The detection of compounds was also done under UV (254 nm) light observation.

Flash chromatography was performed on silica gel M60 (0.04-0.063 mm, 230-400 mesh ASTM) (Macherey-Nagel) under pressure with the eluent mentioned in the respective procedures.

Analytical techniques and devises

GC-MS

GC-MS was performed on HP 6890 gas chromatograph coupled to an MSD 5973 (EI 70 eV) (Hewlett Packard) and on a GC 7890A coupled to an MSD 5975C (Agilent Technologies). Separation was performed on a fused-silica capillary columns BPX-5 (SGE Inc., 25 m x 0.22 mm I.D. x 0.25 µm) and HP5-MS (Agilent Technologies, 30 m x 0.25 mm I.D. x 0.25 µm). Instrument parameters were adjusted as follows: inlet pressure 77.1 kPa; He 23.3 ml/min; injector 250 °C; injection volume 1 µl; transfer line 300°C; electron energy 70eV. Synthetic samples were analyzed in split mode (ratio 20:1) whereas the injector was operated in split-less mode (60 s valve time) for the investigation of natural extracts. Retention indices of natural compounds under non-isothermal conditions were determined from a homologous series of *n*-alkanes. Compounds were identified by comparison of their mass spectra with those from NIST mass spectral library (version 2.0, 23 July 2008) or synthetic references.

Chiral GC for compound 5

Chiral gas chromatography was performed using a hydrodex-6-TBDMS phase (Macherey-Nagel, 25 m x 0.25 mm I.D.). Temperature program: 50°C for 5 min, then with 0.2°C/min to 200°C/min. Hydrogen was applied as carrier gas and compounds were detected with a flame ionization detector. Injector temperature 250°C; detector temperature 300°C; injection volume 1 µl; inlet pressure 80.0 kPa; carrier gas flow 1 mL/min; gas velocity 29 cm/sec.

^{1}H NMR and ^{13}C NMR

NMR spectra were obtained with the following instruments: BRUKER DPX-200 (^{1}H 200 MHz, ^{13}C 50.5 MHz), DRX-400 (^{1}H 400 MHz, ^{13}C 101 MHz) or AV II-600 (^{1}H 600 MHz, ^{13}C 151 MHz). Chemical shifts are reported in ppm relative to tetramethylsilane as an internal standard (δ = 0). All compounds were dissolved in the respective deuterated solvents mentioned.

Derivatization procedure

About 50 µL of MSTFA was added to the natural extract or the synthetic sample in dichloromethane (50 µL), followed by heating for 1h at 60°C.[173] Then excess solvent

and MSTFA was removed by a gentle stream of nitrogen. The residue was taken up in dichloromethane and analyzed by GC-MS.

8.2 General procedures.

A. Diesterification with methanol and HCl.

A solution of corresponding acid compound (with two acid groups) dissolved in an appropriate amount of anhydrous methanol was treated at room temperature with concentrated HCl (0.3 ml). This reaction mixture was heated to reflux for 15 h under inert conditions and vigorous stirring.[64] After completion of the reaction excess of methanol was removed under *vacuo* and the crude product was purified by column chromatography over silica gel with an eluent mixture containing hexane and ethyl acetate in the ratio of 2:1 to yield pure product.

B. Methylation of the diester with lithium diisopropyl amide (LDA).

Generation of LDA

To a one fold amount (in mL) of diisopropylamine in anhydrous THF four a fold amount (in mL) of *n*-BuLi (1.6M in hexane) was added slowly under stirring using a syringe pump over a period of 15 minutes.[64] After completion of the addition the reaction mixture was allowed to stir for further 20 minutes yielding LDA. The reaction mixture was maintained throughout under inert atmosphere and at -78°C.

Methylation

To the above prepared LDA solution 1 equivalent amount of diester in anhydrous THF was added using a syringe pump over a period of 10 min. The resulting solution was stirred for 15 min at –78°C and then slowly warmed to –20°C over a period of 2 h, stirred for 20 min at this temperature and re-cooled to –78°C. Then 1.5 equivalents methyl iodide were slowly added. The reaction mixture was allowed to stir for 30 min at this temperature and slowly warmed to room temperature over a period of 3 h. Stirring was continued for 1 h, followed by quenching the reaction mixture with 10%

citric acid in water and extraction with ethyl acetate (x3). The combined organic phases were washed with brine and water, dried with magnesium sulfate and concentrated in *vacuo*. The crude product was purified by column chromatography on silica gel using a solvent mixture of hexane and ethyl acetate in a ratio of 4:1 to yield the desired product.

C. Formation of acid chlorides

To a 1 molar equivalent solution of the respective acid in anhydrous ether was added 2 molar equivalents of oxalylchloride over a period of 20 minutes using syringe pump under ice-bath temperature, inert conditions, and stirring. The resulting reaction mixture was allowed to stir for a period of 26 h at room temperature after which the remaining oxalylchloride was removed by fractional distillation, followed by purification on silica gel using 2:1 mixture of pentane / diethyl ether as eluent.[103]

D. Coupling of acid chlorides with the chiral auxiliary (*S*)-4-(phenylmethyl)-2-oxazolidinone

To a flask equipped with a magnetic stirring bar charged with 1.1 equivalents of (*S*)-4-(phenylmethyl)-2-oxazolidinone, capped with a rubber septum, and flushed with nitrogen, anhydrous tetrahydrofuran is then added to the flask via syringe, and the resulting solution is cooled to −78°C in an acetone–dry ice bath. To the resulting mixture a solution of 1 equivalent *n*-BuLi (1.6M in hexane) is dropped over a period of 15 minutes by means of a syringe pump, followed by addition of the respective acid chloride to the reaction mixture dissolved in a minimum amount of anhydrous THF. The resulting clear, nearly colorless solution was stirred for 3h at −78°C, and then allowed to warm to room temperature over a period of 30 minutes.[104,105] Excess of acid chloride is quenched by the addition of saturated aqueous ammonium chloride. The bulk of the tetrahydrofuran and hexane were removed on a rotary evaporator, and the resulting slurry was extracted with dichloromethane. The combined organic extracts were washed with 1M aqueous sodium hydroxide solution and saturated sodium chloride solution, dried with magnesium sulfate, and filtered. The solvent is removed by rotary evaporation, and the crude product was purified by column

chromatography on silica gel with an eluent mixture containing pentane and diethyl ether in the ratio of 5:1 to yield pure product.

E. Stereo-selective methylation of Evans chiral auxiliary acid derivatives

To a solution of 1 molar equivalent chiral acid derivative in appropriate amount of anhydrous THF was added drop-wise 1.1 equivalents of Na-HMDS dissolved in a anhydrous THF over a period of 10 minutes using syringe pump under nitrogen atmosphere and at -78°C. The resulting reaction mixture was stirred at -78°C for 1h, and then 2 equivalents of iodomethane were added drop-wise. The reaction mixture was stirred for 4h at -78°C, followed by the addition of saturated ammonium chloride solution and water.[105] The resulting aqueous phase was acidified to pH = 2 with 2M sulfuric acid. The product was extracted with ethyl acetate (x3) and the combined extracts were successively washed with saturated sodium hydrogen carbonate solution, saturated sodium thiosulfate solution and brine, dried with magnesium sulfate, filtered, and concentrated in *vacuo* to give an oil which was purified by column chromatography over silica gel using 5:1 mixture of pentane/ethyl acetate as eluent.

F. Chiral auxiliary removal

A solution of 5 molar equivalent 30% hydrogen peroxide and 2 equivalents of lithium hydroxide monohydrate dissolved in water were added successively to a solution of a methylated chiral acid derivative in a THF/water mixture (2:1) at 0°C. After 2h stirring, aqueous sodium sulfite solution was added and the resulting mixture was stirred for 15 minutes at 0°C. Then the pH of the solution was adjusted between 9-10 using saturated sodium hydrogencarbonate solution. THF was evaporated and the residual aqueous solution was extracted with dichloromethane (x3). The organic extracts were dried with magnesium sulfate and evaporation of solvent yielded the cleaved chiral auxiliary in pure form. The remaining aqueous solution was acidified to pH 1-2 with 1M sulfuric acid and was extracted with diethyl ether (x3). The combined organic extracts were dried with magnesium sulfate, filtered and concentrated in *vacuo* to give the acid.[107]

G. Esterification of acids with EDC

Equi-molar quantities of acid and alcohol were dissolved in anhydrous dichloromethane (in case of methanol no solvent was used), cooled to 0°C, followed by the addition of in 0.1 equivalent DMAP. Under nitrogen atmosphere 1.1 molar equivalents EDC were added in small portions to the reaction mixture.[79] This mixture was allowed to stir at 0°C for 1h followed by overnight stirring at room temperature, after which water and dichloromethane were added, and the organic phase was separated. The aqueous layer was washed twice with dichloromethane. The separated organic phases were combined, dried with magnesium sulfate, and filtered. After removal of solvent under *vacuo*, the crude product was purified by column chromatography on silica gel with an eluent mixture mentioned.

H. Esterifications with boron trifluoride diethyl etherate

To a 1 equivalent solution of the respective acid in absolute methanol was added 1 equivalent solution of boron trifluoride diethyl etherate under ice-bath cooling and nitrogen atmosphere. The resulting reaction mixture was stirred over night at room temperature followed by the addition of water and sodium hydrogen carbonate.[108] The aqueous phase was extracted with diethyl ether (x3), and the combined extracts were dried with magnesium sulfate, filtered, and concentrated in *vacuo* to give the crude product which was purified by column chromatography over silica gel using the eluent mentioned.

I. Reduction of esters with lithium aluminium hydride ($LiAlH_4$)

A suspension of 4 equivalents of lithium aluminium hydride in anhydrous diethyl ether was prepared and cooled to 0° C under nitrogen atmosphere, followed by the addition of 1 equivalent of the corresponding ester in anhydrous diethyl ether.[136] The suspension was allowed to warm up to room temperature and the progress of the reaction was controlled by TLC. After the consumption of the educt was completed, a 2M aqueous HCl-solution was added till two clear phases were formed and the organic phase was separated. The aqueous layer was extracted twice with diethyl ether and the organic phases were combined. The combined organic phase was

dried with $MgSO_4$ and the solvents were removed under reduced pressure. Crude products were purified by column chromatography on silica gel with a mixture of eluent mentioned.

J. Oxidation of alcohols to aldehydes

A solution of 1.2 equivalents of oxalyl chloride was added over 10 minute period to a solution of 2.5 equivalents dimethyl sulfoxide in anhydrous dichloromethane at -78°C under nitrogen atmosphere. The reaction mixture was stirred for 20 minutes followed by the addition of 1 equivalent alcohol dissolved in a minimum amount of dichloromethane over a period of 25 minutes by means of syringe pump. After stirring the solution for further 45 minutes at -78°C, 4.5 molar equivalents of triethyl amine was added. The resulting thick white mixture was warmed to room temperature over a period of 1h followed by the addition of water.[111] The aqueous layer was extracted with dichloromethane (x3). The separated organic phases were combined, washed with water and saturated sodium chloride, dried with magnesium sulfate, and filtered. After removal of solvent the under *vacuo*, the crude product was purified by column chromatography on silica gel with an eluent mixture mentioned.

K. Generation of Wittig salt from the corresponding bromides

Equi-molar quantities of triphenyl phosphine and the corresponding bromide were dissolved in anhydrous toluene under nitrogen atmosphere and magnetic stirring. The resulting reaction mixture was heated to reflux for 48h. After completion of the reaction, excess of solvent was removed under rotary evaporator and the remaining white solid was dissolved in dichloromethane, followed by precipitating it from cold diethyl ether.[139]

L. Formation of alkene by Wittig reaction

A solution of 1 equivalent Wittig salt in anhydrous dimethoxyethane (DME) was cooled to -78°C, to which 2.5 molar equivalents of 1 *M* Li-HMDS solution in THF was added drop-wise under nitrogen atmosphere. The mixture was allowed to warm up to room temperature and stirring was continued for 30 minutes. Then, the mixture was re-cooled to -78°C, followed by the addition of 1 equivalent of aldehyde component. The cooling bath was removed and stirring was continued overnight at room temperature.[138] After completion of the reaction, the mixture was quenched with 2M aqueous HCl solution, followed by the addition of diethyl ether. The organic phase was separated and the aqueous phase was washed twice with diethyl ether. The organic phases were combined, dried with magnesium sulfate, and the solvents were removed under reduced pressure yielding crude product that was purified by column chromatography on silica gel with an eluent mixture mentioned.

M. Formation of Iodides from corresponding alcohols

An equi-molar suspension of triphenyl phosphine and imidazole in a solvent mixture containing anhydrous acetonitrile and anhydrous diethyl ether in a ratio of 3:1 was prepared and cooled to 0°C under nitrogen atmosphere, to which an equi-molar quantity of iodine crystals were added in portions. The mixture was stirred for 30 minutes at room temperature, followed by re-cooling to 0°C. The corresponding alcohol in 0.5 equi-molar quantity was added dissolved anhydrous diethyl ether.[140] The resulting reaction mixture was allowed to warm up to room temperature and stirring was continued overnight. After completion of the reaction aqueous saturated solution of sodium hydrogen carbonate was added and the organic phase was separated. The aqueous phase was washed twice with diethyl ether. The organic phases were combined, dried with magnesium sulfate, and the solvents were removed under reduced pressure. The crude product was purified by column chromatography on silica gel with an eluent mixture mentioned.

N. Formation of nitriles from corresponding iodide

To a 1 molar equivalent solution of corresponding iodide dissolved in an appropriate amount of anhydrous DMSO, a solution of 2 equivalents tetraethylammonium cyanide in DMSO was added under nitrogen atmosphere. The progress of the reaction was controlled by TLC. After 2h the educt was completely consumed. The reaction mixture was quenched with water and diethyl ether. The organic phase was separated and the aqueous phase was extracted twice with diethyl ether. The combined organic phases were dried with magnesium sulfate and the solvents were removed under *vacuo*. The crude product was purified by column chromatography on silica gel with the eluent mixture mentioned.[141]

O. Formation of acids from corresponding nitriles

To a solution of alkyl/alkenyl nitrile in an appropriate amount of 60 % aqueous ethanol, 20 equivalents of sodium hydroxide were added and the resulting reaction mixture was heated to reflux for 24h. After completion, the reaction mixture was treated with water and concentrated HCl.[142] The aqueous phase was extracted thrice with diethyl ether. The separated organic phases were combined, washed with water and saturated sodium chloride, dried with magnesium sulfate, and filtered. After removal of solvent under *vacuo*, the crude product was purified by column chromatography on silica gel with an eluent mixture mentioned.

P. Hydrogen/deuterium exchange at the α-position of methyl carboxylates

An excess amount of [D_1]-methanol was used to dissolve 15 molar equivalents of freshly divided sodium metal to which 1 molar equivalent of the corresponding carboxylic ester was added, dissolved in a minimum amount of [D_1]-methanol. The resulting reaction mixture was heated to reflux for 6h, followed by diluting the mixture with diethyl ether and adding 2M aqueous HCl.[168] The organic phase was separated and the aqueous phase was extracted twice with diethyl ether. The combined organic phases were dried with magnesium sulfate, and the solvents were removed under reduced pressure yielding a product mixture of α-deuterated ester and the

corresponding free acid which was subsequently purified by column chromatography on silica gel with an eluent mixture mentioned.

8.3 Synthesis of reference compounds

8.3.1 Synthesis of racemic trimethyl methylcitrate (13)

(*S*)-Dimethyl malate (21)

This compound was synthesized according to the general procedure **A**.

Amounts used: 6.3 g (47 mmol) of **20**, 40 ml of anhydrous methanol.

Yield: 6.7 g (88%).

R_f = 0.34 (hexane/ethyl acetate 2:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 2.73-2.85 (m, 1H, CH_2), 2.85-2.96 (m, 1H, CH_2), 3.72 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 4.53 (dd, J = 5.8, 4.6 Hz, 1H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 39.1(CH_2), 52.6 (OCH_3), 53.4 (OCH_3), 67.8 (C-OH), 171.6 (CO), 174.3 (CO). EI-MS (70 eV): *m/z* (%): 163 (0.4) $[M+1]^+$, 131 (7), 103 (100), 71 (78), 61 (37), 43 (61).

Dimethyl (2*S*,3*R*)-3-methylmalate (22)

This compound was synthesized according to the general procedure **B**.

Amounts used for LDA generation: 9.3 ml of diisopropylamine (66 mmol, 6.7 g), 30 ml of anhydrous THF (30 ml), 38 ml of *n*-BuLi (1.6M in hexane)

Amounts used for alkylation: 4.26 g (26.3 mmol) of **21**, 5 ml of anhydrous THF, 5.6 g (40 mmol) of methyl iodide.

Yield: 3.7 g (80%).

R_f = 0.34 (hexane/ethyl acetate 4:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.30 (d, J = 7.3Hz, 3H, CH_3), 3.05 (qd, J = 7.2, 3.8 Hz, 1H, $CHCH_3$), 3.70 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 4.29 (dd, J = 6.3, 3.8 Hz, 1H, CH). ^{13}C NMR (50 MHz, $CDCl_3$): δ = 12.9 (CH_3), 43.0 ($CHCH_3$), 51.8 (OCH_3), 52.5(OCH_3), 72.3 (CH), 173.2 (CO), 173.5 (CO).

EI-MS (70 eV): *m/z* (%): 177 (0.3) $[M+1]^+$, 161 (0.4), 145 (6), 117 (100), 85 (98), 57 (66), 61 (21), 88 (19).

Methyl 2-hydroxyacetate (24)

A solution of **23** (4 g, 52.6 mmol) in absolute methanol (80 ml) was treated with concentrated HCl (0.3ml) at room temperature, followed by heating to reflux for 15h. After the completion of the reaction excess of methanol was removed under *vacuo*, and the crude product was purified by column chromatography using 2:1 mixture of pentane/diethyl ether as eluent.[65]

Yield: 3.5 g (74%)

R_f = 0.80 (pentane/diethyl 2:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 3.77 (s, 3H, OCH_3), 4.01 (s, OH), 4.19 (d, J = 4.79 Hz, 2H, CH_2). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 52.11 (OCH_3), 60.63 (CH_2), 173.92 (CO).

Methyl 2-iodoacetate (25)

This compound was synthesized according to the general procedure **M**.

Amounts used: 3.3 g (36.6 mmol) of **24** dissolved in 8 ml of diethyl ether, 9.6 g (36.6 mmol) of triphenyl phosphine, 2.49 g (36.6 mmol) of imidazole, 9.3 g (36.6 mmol) of iodine, 80 ml of 3:1 mixture of diethyl ether and acetonitrile.[66-69]

Yield: 4.74 g (65%)

R_f = 0.48 (hexane/ethyl acetate 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 3.71 (s, 2H, CH_2) 3.76 (s, 3H, OCH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ : -6.19 (CH_2), 65.76 (OCH_3), 169.19 (CO).

Racemic trimethyl methylcitrate (13)

Li-HMDS (8 ml, 1.0 M in hexane) was added drop-wise to a stirred solution of compound **22** (2 g, 11 mmol) in dry THF (50 ml) at -78°C under nitrogen atmosphere

over a period of 10 minutes using syringe pump. The reaction mixture was stirred for further 20 minutes at -78°C followed by the drop-wise addition of compound **25** (4.74g, 23.8 mmol) dissolved in minimum amount of anhydrous THF (~2 ml). The resulting mixture was stirred for 3h at -78°C, and was then allowed to rise to room temperature within the next 1h. Work up was carried out by acidifying the solution to pH=2 followed by extraction with dichloromethane (x3). The organic phases were combined, dried over magnesium sulfate, filtered, concentrated under *vacuo*, and the crude product was purified by column chromatography using 1:1 eluent mixture containing hexane/ethyl acetate.

Yield: 0.338 g (12%).

R_f = 0.80 (hexane/ethyl acetate 1:1). ^{1}HMR (600 MHz, $CDCl_3$) δ: 1.25 (d, J = 7.2 Hz, 3H, CH_3), 2.90 (d, J = 16.3 Hz, 1H, CH_2), 2.95 (q, J = 7.2 Hz, 1H, CH), 3.12 (d, J = 16.3 Hz, 1H, CH_2), 3.69 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3). ^{13}C NMR (600 MHz, $CDCl_3$) δ: 11.25 (CH_3), 40.4 (CH_2), 46.2 (CH), 52.0 (OCH_3), 52.1 (OCH_3), 53.0 (OCH_3), 75.9 (C-OH), 170.9 (CO), 173.6 (CO), 174.3 (CO). EI-MS (70 eV): *m/z* (%): 157 (100), 115 (51), 59 (34), 101 (25), 88 (21), 56 (15), 57 (13), 43 (12), 189 (11), 125 (8).

8.3.2 Synthesis of trimethyl (2*R*,3*S*)-methylcitrate (13c)

2-((2*S*,4*S*)-2-*tert*-Butyl-5-oxo-1,3-dioxolan-4-yl)acetic acid (26)

Concentrated sulfuric acid (0.3 ml) was added to a suspension of (*S*)-malic acid (**20**) (10 g, 75 mmol), pivalaldehyde (10g, 116 mmol), and *p*-toluenesulphonic acid (1.25 g, 6.5 mmol) in pentane (125 ml). The reaction mixture was heated to reflux for 4h in a flask that was connected to a water separator. After completion of the reaction, the mixture was allowed to cool resulting in a solid which was dissolved in dichloromethane. The resulting organic phase was washed (x2) with 10% phosphoric acid, dried with magnesium sulfate, and filtered.[75] The resulting solution was concentrated to half of its volume and placed in the refrigerator at -18°C allowing the crystallization forming colorless crystals of compound **26**.

Yield: 9.19 g (61%).

Fp = 107°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.98 (d, J = 3.1 Hz, 9H, $C(CH_3)_3$), 2.77-2.94 (dd, J=7.6 Hz, J=5.9 Hz, 1H, CH_2), 3.01-3.05 (dd, J = 3.6 Hz, J = 2.6 Hz 1H,

CH_2), 4.63-4.68 (dd, *J* = 2.6 Hz *J* = 1.0 Hz, 1H, CH), 5.20 (m, 1H, CH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 22.97 ((CH_3)$_3$), 33.81 (C(CH_3)$_3$), 34.85 (CH_2), 71.01 (CH), 109.46 (CH), 171.60 (CO), 173.45 (CO).

(2*S*,3*R*)-3-Methylmalic acid (29)

To a solution of **22** (3,7 g, 21 mmol) in tetrahydrofuran/methanol (1:1, 40 ml) was added 2 N KOH (40 ml, 80 mmol) and the resulting solution was stirred at room temperature for 24 h. Then the reaction mixture was acidified to pH=1 by the addition of concentrated HCl, followed by neutralization with brine.[74] The resulting mixture was extracted with ethyl acetate (x3), followed by drying with magnesium sulfate and concentrating in *vacuo* to yield pure **29**.

Yield: 2.9 g (92%).

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.22 (d, *J* = 7.1 Hz, 3H, CH_3), 2.95 (qd, *J* = 7.2, 5.0 Hz, 1H, CH), 4.32 (d, *J* = 5.0 Hz, 1H, CH_2). ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 13.3 (CH_3), 44.6 (CH), 73.5 (CH_2), 175.2 (CO), 176.7 (CO). EI-MS (70 eV) (MSTFA derivative): *m/z* (%): 364 (0.1) $[M]^+$, 349 (17), 247 (65), 189 (17), 147 (93), 115 (25), 73 (100).

(*R*)-2-((2*S*,4*S*)-2-*tert*-Butyl-5-oxo-1,3-dioxolan-4-yl)propanoic acid (30)

Pivalaldehyde (2.5 g, 29 mmol) and *p*-toluenesulphonic acid (1.25 g, 6.5 mmol) were added to a stirred suspension of **29** (2.9 g, 19.5 mmol) in pentane (50 ml), followed by the addition of concentrated sulfuric acid (0.3 ml). The reaction was heated to reflux for 4h in a flask containing a water separator. Dichloromethane was added and the organic phase was washed with 10% phosphoric acid.[75] Finally the volume of the organic phase was reduced to one fourth and cooled down to -18°C. Crystallization occurred overnight to form colorless crystals of pure **30**.

Yield: 2.1 g (50%).

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.96 (d, *J* = 6.8 Hz, 9H, (CH_3)$_3$), 1.38 (dd, *J* = 7.3, 3.0 Hz, 3H, CH_3), 3.03 (qd, *J* = 7.3, 4.8 Hz, 1H, CH), 4.41 (ddd, *J* = 9.9 Hz, *J* = 4.6 Hz, *J* = 1.5 Hz, 1H, CH), 5.29-5.36 (m, 1H, CH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 12.8 (CH_3),

23.2 ($(CH_3)_3$), 34.2 ($C(CH_3)_3$), 40.4 (CH), 76.3 (CH), 109.4 (CH), 172.3 (CO), 178.1 (CO). EI-MS (70 eV): *m/z* (%): 216 (0.1) $[M]^+$, 170 (6), 159 (20), 131 (10), 103 (66), 85 (65), 70 (100), 57 (55).

(*R*)-2-((2*S*,4*S*)-4-Allyl-2-*tert*-butyl-5-oxo-1,3-dioxolan-4-yl)propanoic acid (31)

Li-HMDS (21 ml, 1.0 M in hexane) was added using a syringe pump over a period of 10 minutes to a stirred solution of **30** (2.1 g, 9.7 mmol) in dry THF (120 ml) under a nitrogen atmosphere at -78°C. The resulting mixture was allowed to stir at -78°C for further 20 minutes, followed by the addition of freshly distilled allyl bromide (2.6 g, 21.6 mmol). Stirring was continued for 3h at –78°C, followed by a slow temperature raise to room temperature. Workup was carried out by adding 1 M HCl until a pH of 2-3 was reached, followed by extractions with dichloromethane (x3).[75] The combined organic phases were dried with magnesium sulfate and the solvent evaporated in *vacuo* to yield crude **31**. Purification of this compound proved to be difficult, so it was used in the next step without purification.

Yield: 1.96 g (79%).

EI-MS (70 eV): *m/z* (%): 256 (0.1) $[M]^+$, 215 (6), 202 (7), 185 (5), 184 (4), 157 (13), 156 (27), 139 (30), 57 (100).

Dimethyl (2*S*,3*R*)-2-allyl-2-hydroxy-3-methylsuccinate (32):

To an ice cooled solution of **31** (1.95 g, 7.6 mmol) in dry methanol (30 ml) 5 equivalents of boron trifluoride diethyl etherate (5.7 g, 40 mmol) were added using a syringe pump over 15 min. The reaction mixture was allowed to stir for 48h followed by neutralization with 1N sodium hydroxide. The reaction mixture was extracted with ethyl acetate (x5). The combined organic phases were washed with brine, dried with magnesium sulfate, and the solvent was removed in *vacuo* giving the crude product **32** that was directly used in the next reaction.

Yield: 0.97 g (59%)

EI-MS (70 eV): *m/z* (%): 216 (0.1) $[M]^+$, 215 (2), 199 (25), 175 (4), 157 (100), 129 (2), 87 (13), 59 (22), 57 (20).

(3*S*,4*R*)-3-Hydroxy-5-methoxy-3-(methoxycarbonyl)-4-methyl-5-oxopentanoic acid (33)

Ruthenium trichloride hydrate (24 mg, 0.105 mmol) was added to a suspension of **32** (0.9 g, 4.16 mmol), followed by the addition of sodium metaperiodate (4 g, 18.75 mmol) in carbon tetrachloride (10 ml), acetonitrile (10 ml), and water (15 ml). The reaction mixture was allowed to stir for 3 days. Then dichloromethane and saturated sodium chloride solution were added. The organic phase was separated and the aqueous phase was washed with dichloromethane (x3). The combined organic phases were dried with magnesium sulfate and the volume was reduced to 15 ml. The ruthenium compounds were removed by performing column chromatography on silica gel (diethyl ether / hexane / acetic acid 20:15:0.3) to yield pure **33**.[77,78]

Yield: 0.161 g (18%).

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.21 (d, *J* = 7.3 Hz, 3H, CH_3), 2.86 (d, *J* = 15.1 Hz, 1H, CH_2), 2.98 (d, *J* = 15.1 Hz, 1H, CH_2), 3.20 (q, *J* = 7.8 Hz, 1H, CH), 3.61 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3). ^{13}C- NMR (50 MHz, $CDCl_3$): δ = 11.4 (CH_3), 40.8 (CH_2), 43.6 (CH), 52.9 (OCH_3), 54.7 (OCH_3), 74.2 (C-OH), 174.5 (CO), 175.2 (CO), 176.8 (CO).

Trimethyl (2*R*,3*S*)-methylcitrate (13c)

This reaction was performed according to the general procedure **G**.

Amounts used: 0.150 g (0.64 mmol) of the acid **33**, 25 ml of absolute methanol, 0.151 g (0.79 mmol) of 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), and 0.0065 g (0.054 mmol) of 4-(N,N-dimethylamino)pyridine (DMAP).

Yield: 0.090 g (57%).

(2*R*,3*S*) Isomer. R_f = 0.80 (hexane/ethyl acetate 1:1). ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 1.26 (d, *J* = 7.2 Hz, 3H, CH_3), 2.91 (d, *J* = 16.3 Hz, 1H, CH_2), 2.96 (q, *J* = 7.2 Hz, 1H,

CH), 3.15 (d, *J* = 16.3 Hz, 1H, CH), 3.69 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 11.25 (CH_3), 40.4 (CH_2), 46.2 (CH), 52.0 (OCH_3), 52.1 (OCH_3), 53.0 (OCH_3), 75.9 (C-OH), 170.9 (CO), 173.6 (CO), 174.3 (CO). EI-MS (70 eV): *m/z* (%): 157 (100), 115 (51), 59 (34), 101 (25), 88 (21), 56 (15), 57 (13), 43 (12), 189 (11), 125 (8).

(2*S*,3*S*) Isomer. R_f = 0.80 (hexane/ethyl acetate 1:1). ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 1.21 (d, *J* = 7.2 Hz, 3H, CH_3), 2.85 (d, *J* = 16.3 Hz, 1H, CH_2), 2.87 (q, *J* = 7.2 Hz, 1H, CH), 3.05 (d, *J* = 16.3 Hz, 1H, CH_2), 3.69 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 12.2 (CH_3), 41.0 (CH_2), 47.1 (CH), 52.2 (OCH_3), 52.2 (OCH_3), 53.2 (OCH_3), 75.9 (C-OH), 171.1 (CO), 173.2 (CO), 173.9 (CO). EI-MS (70 eV): *m/z* (%): 157 (100), 115 (51), 59 (34), 101 (25), 88 (21), 56 (15), 57 (13), 43 (12), 189 (11), 125 (8).

8.3.3 Synthesis of undecyl (*S*)-2-methyltridecanoate (*S*)-47

Tridecanoyl chloride (53)

This reaction was performed according to the general procedure **C**.

Amounts used: 2.0 g (9.3 mmol) of the acid **52**, 20 ml of anhydrous diethyl ether, 2.37 g (18.6 mmol) of oxalyl chloride.

Yield: 1.97 g (91%).

R_f = 0.56 (pentane/diethyl ether 2:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.88 (t, *J* = 6.2 Hz, 3H, CH_3), 1.26-1.31 (m, 18H, 9x CH_2), 1.80 (quin, *J* = 7.23 Hz, 2H, CH_2), 2.91 (t, *J* = 5.6 Hz, 2H, CH_2). ^{13}C- NMR (50 MHz, $CDCl_3$) δ: 14.16 (CH_3), 21.7 (CH_2), 25.7 (CH_2), 28.6 (CH_2), 28.8 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.6 (CH_2), 29.8 (CH_2), 31.7 (CH_2), 46.4 (CH_2), 174.2 (CO). EI-MS (70 eV): *m/z* (%): 232 (1%) $[M]^+$, 197 (37), 152 (15), 112 (34), 98 (100), 84 (65), 69 (29), 55 (47), 43 (23).

(*S*)-4-benzyl-3-tridecanoyloxazolidin-2-one ((*S*)-54)

This reaction was performed according to the general procedure **D**.

Amounts used: 1.9 g (8.2 mmol) of the acid chloride **53**, 1.59 g (9 mmol) of (*S*)-4-(phenylmethyl)-2-oxazolidinone, 30 ml of anhydrous tetrahydrofuran, 8 ml of *n*-Buli (1.6M in hexane).

Yield: 0.9 g (30%).

R_f = 0.20 (pentane/diethyl ether 5:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.88 (t, *J* = 6.80 Hz, 3H, CH_3), 1.21-1.31 (m, 16H, 8x CH_2), 1.43 (quin, *J* = 7.7 Hz, 2H, CH_2), 1.58 (quin, *J* = 7.7 Hz, 2H, CH_2), 2.77 (dd, *J* = 13.3 Hz, *J* = 9.6 Hz, 2H, CH_2), 3.25 (t, *J* = 7.1 Hz, 2H, CH_2), 4.09-4.22 (m, 2 H, CH_2), 4.65-4.71 (ddt, *J* = 10.61, *J* = 6.71, *J* = 3.28, 1H, CH), 7.21-7.23 (m, 2H, 2xCH), 7.27-7.29 (m, 1H, CH), 7.31-7.35 (m, 2H, 2xCH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.1 (CH_3), 22.6(CH_2), 24.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 31.8 (CH_2), 31.9 (CH_2), 33.4(CH_2), 35.43 (CH_2) 37.7 (CH), 37.9 (CH_2), 55.3 (CH), 66.0 (CH_2), 127.3 (CH), 128.9 (CH), 129.4 (CH), 129.5 (CH), 129.6 (CH), 135.3 (CH), 153.1 (CO), 177.3 (CO). EI-MS (70 eV): *m/z* (%): 373 (4) $[M]^+$, 282 (8), 232 (5), 219 (10), 197 (100), 178 (7), 134 (6), 117 (10), 91 (9), 71 (8), 57 (12).

(*S*)-4-Benzyl-3-((*S*)-2-methyltridecanoyl) oxazolidin-2-one ((*S*,*S*)-55)

This reaction was performed according to the general procedure **E**.

Amounts used: 0.75 g (2.0 mmol) of the Evans chiral auxiliary acid derivative (***S***)-**54**, 6 ml of anhydrous tetrahydrofuran, 5 ml of Na-HMDS (1.0M in hexane) in 15 ml of anhydrous tetrahydrofuran, 0.568 g (4 mmol) of iodomethane.

Yield: 0.526 g (68%). (d.r. 97:1)

R_f = 0.45 (pentane/ethyl acetate 5:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.88 (t, *J* = 6.8 Hz, 3H, CH_3), 1.18 (d, *J* = 6.8 Hz, 3H, CH_3), 1.21-1.31 (m, 16H, 8x CH_2), 1.58-1.63 (m, 2H, CH_2), 2.77 (dd, *J* = 13.3 Hz, *J* = 9.6 Hz, 2H, CH_2), 3.25-3.30 (m, 1H), 3.69 (dd, *J* = 6.8 Hz, *J* = 5.2 Hz, 2H, CH_2), 4.09-4.22 (m, 2 H, CH_2), 4.65-4.71 (ddt, *J* = 10.61, *J* = 6.71, *J* = 3.28, 1H, CH), 7.21-7.23 (m, 2H, 2xCH), 7.27-7.29 (m, 1H, CH), 7.31-7.35 (m, 2H, 2xCH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.1 (CH_3), 16.8 (CH_3), 22.6(CH_2), 27.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 31.8 (CH_2), 31.9 (CH_2), 33.4(CH_2), 39.3 (CH), 37.9 (CH_2), 55.3 (CH), 66.0 (CH_2),

127.3 (CH), 128.9 (CH), 129.4 (CH), 129.5 (CH), 129.6 (CH), 135.3 (CH), 153.1 (CO), 177.3 (CO). EI-MS (70 eV): *m/z* (%): 387 (5) $[M]^+$, 296 (8), 233 (26), 212 (16), 211 (100), 183 (15), 178 (20), 134 (8), 117 (17), 99 (12), 71 (24), 57 (22).

(*S*)-2-Methyltridecanoic acid ((*S*)-56)

This reaction was performed according to the general procedure **F**.

Amounts used: 0.5 g (1.3 mmol) of the (*S*)-4-benzyl-3-((*S*)-2-methyltridecanoyl) oxazolidin-2-one ((***S***,***S***)-**55**) dissolved in 30 ml of 2:1 THF/ H_2O mixture, 5 ml of 30% H_2O_2 solution, 0.6 g (2.6 mmol) lithium hydroxide monohydrate dissolved in 5 ml of water.

Yield: 0.200 g (69%).

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.88 (t, *J* = 6.80 Hz, 3H, CH_3) 1.18 (d, *J* = 6.8 Hz, 3H, CH_3), 1.21-1.31 (m, 16H, 8xCH_2), 1.37 (m, 2H, CH_2), 1.45 (q, *J* = 9.8 Hz, 2H, CH_2), 2.43 (m, 1H, CH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.1 (CH_3), 16.8 (CH_3), 22.6(CH_2), 27.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 31.8 (CH_2), 31.9 (CH_2), 33.4(CH_2), 39.3 (CH), 183.4 (CO). EI-MS (70 eV) (MSTFA derivative): *m/z* (%): 300 (5) $[M]^+$, 285 (100), 159 (11), 147 (10), 146 (82), 143 (28), 130 (25), 117 (13), 73 (91).

Undecyl (*S*)-2-methyltridecanoate (*S*)-47

This reaction was performed according to the general procedure **G**.

Amounts used: 0.2 g (0.8 mmol) of the acid (***S***)-**56**, 28 ml of anhydrous dichloromethane, 0.164 g (0.8 mmol) of undecanol, 0.01 g (0.08 mmol) DMAP, 0.185 g (0.96 mmol) of 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC).

Yield: 0.150 g (49%).

R_f = 0.81 (pentane/diethylether 10:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.88 (t, *J* = 6.80 Hz, 6H, 2xCH_3) 1.12 (d, *J* = 7.1 Hz, 3H, CH_3), 1.28-1.34 (m, 34H, 17xCH_2), 1.61 (q, *J* = 13.6 Hz, 2H, CH_2), 2.2 (quin, *J* = 6.9 Hz, 2H, CH_2), 2.6 (m, 1H, CH), 4.05 (t, *J* = 6.1 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.0 (2xCH_3), 17.1 (CH_3), 22.6 (2xCH_2),

25.9 (CH_2), 27.2 (CH_2), 28.6 (CH_2), 29.2 (CH_2), 29.3 (2xCH_2), 29.4 (CH_2), 29.5 (2xCH_2), 29.6 (2xCH_2), 29.7 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 31.9 (2xCH_2), 33.8 (CH_2), 39.6 (CH), 64.2 (OCH_2), 177.0 (CO). EI-MS (70 eV): *m/z* (%): 382 (4) $[M]^+$, 229 (100), 211 (4), 199 (14), 154 (27), 126 (7), 111 (13), 97 (14), 83 (14).

8.3.4 Synthesis of heptadecyl (*S*)-4-methylheptanoate (*S*)-50

Pentanoyl chloride (58)

This reaction was performed according to the general procedure **C**.

Amounts used: 5.0 g (49 mmol) of the acid **57**, 100 ml of anhydrous diethyl ether, 12.4 g (98 mmol) of oxalyl chloride.

Yield: 4.92 g (83%).

R_f = 0.52 (pentane/diethyl ether 2:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.96 (t, *J* = 7.0 Hz, 3H, CH_3), 1.32 (sext, *J* = 7.3 Hz, 2H, CH_2), 1.61 (q, *J* = 8.3 Hz, 2H, CH_2), 2.81 (t, *J* = 7.33 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 13.8 (CH_3) 22.2 (CH_2) 26.3 (CH_2) 46.1 (CH_2) 173.4 (CO). EI-MS (70 eV): *m/z* (%): 120 (1) $[M]^+$, 85 (100), 78 (33), 57 (93), 55 (44), 41 (80), 29 (80), 27 (60).

(*S*)-4-benzyl-3-pentanoyloxazolidin-2-one ((*S*)-59)

This reaction was performed according to the general procedure **D**.

Amounts used: 4.9 g (40 mmol) of the acid chloride **58**, 7.76 g (44 mmol) of (*S*)-4-(phenylmethyl)-2-oxazolidinone*,* 150 ml of anhydrous tetrahydrofuran, 30 ml of *n*-BuLi (1.6M in hexane).

Yield: 4.17 g (40%).

R_f = 0.26 (pentane/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.95 (t, *J* = 7.0 Hz, 3H, CH_3), 1.32 (sext, *J* = 7.3 Hz, 2H, CH_2), 1.61 (q, *J* = 8.3 Hz, 2H, CH_2), 2.88-2.99 (m, 2H, CH_2), 3.20 (t, *J* = 7.1 Hz, 2 H, CH_2), 4.12-4.25 (m, 2H, CH_2), 4.61-4.73 (m, 1H, CH), 7.18-7.23 (m, 2H, CH), 7.26-7.30 (m, 1H, CH), 7.31-7.38 (m, 2H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 13.8 (CH_3) 22.2 (CH_2) 26.5 (s, 1 C) 35.2 (s, 1 C) 37.9

(s, 1 C) 55.1 (CH) 66.1 (CH_2O) 127.3 (CH) 128.9 (2xCH) 129.3 (2xCH) 135.3 (CH) 153.4 (COO)) 173.4 (CON). EI-MS (70 eV): *m/z* (%): 261 (23) $[M]^+$, 232 (5), 219 (13), 176 (6), 170 (47), 134 (7), 117 (14), 92 (10), 91 (29), 86 (15), 85 (100), 57 (44).

(*S*)-4-Benzyl-3-((*S*)-2-methylpentanoyl) oxazolidin-2-one ((*S*)-(*S*)-60)

This reaction was performed according to the general procedure **E**.

Amounts used: 4.1 g (16 mmol) of the Evans chiral auxiliary acid derivative ((***S***)**-59**), 50 ml of anhydrous tetrahydrofuran, 30 ml of Na-HMDS (1.0M in hexane) in 25 ml of anhydrous tetrahydrofuran. 4.49 g (32 mmol) of iodomethane.

Yield: 4.0 g (92%). (d.r. 98:1)

R_f = 0.40 (pentane/ ethyl acetate 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.92 (t, *J* = 6.8 Hz, 3H, CH_3), 1.19 (d, *J*=6.8 Hz, 3H, CH_3), 1.23 (tq, *J* = 7.6 Hz, *J* = 7.60 Hz, 2H, CH_2), 1.68-1.82 (m, 2H, CH_2), 2.76 (dd, *J* = 13.3 Hz, *J* = 9.6 Hz, 2H, CH_2), 3.19-3.34 (m, 1H, CH), 4.07-4.25 (m, 2H, CH_2), 4.68 (ddt, *J* = 9.6 Hz, *J* = 7.0 Hz, *J* = 3.5 Hz, 1H, CH), 7.18-7.23 (m, 2H, CH), 7.26-7.30 (m, 1H, CH), 7.31-7.38 (m, 2H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 14.0 (CH_3), 17.2 (CH_3), 20.3 (CH_2), 35.5 (CH_2), 37.4 (CH), 37.9 (CH_2), 55.3 (CH), 66.0 (CH_2), 127.3 (CH), 128.9 (2xCH), 129.4 (2xCH), 135.3 (C), 153.0 (COO), 177.3 (CON). EI-MS (70 eV): *m/z* (%): 275 (21) $[M]^+$, 233 (18), 184 (57), 178 (18), 134 (8), 117 (24), 99 (100), 91 (37), 86 (26), 71 (73).

(*S*)-2-Methylpentanoic acid ((*S*)-61)

This reaction was performed according to the general procedure **F**.

Amounts used: 4.0 g (14.5 mmol) of the ((*S*)-4-benzyl-3-((*S*)-2-methylpentanoyl) oxazolidin-2-one ((***S***,***S***)**-60**) dissolved in 150 ml of 2:1 THF/H_2O mixture, 40 ml of 30% H_2O_2 solution, 0.670 g (29 mmol) lithium hydroxide monohydrate dissolved in 15 ml of water.

Yield: 1.5 g (89%).

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.92 (t, J = 6.8 Hz, 3H, CH_3), 1.19 (d, J = 7.0 Hz, 3H, CH_3), 1.27-1.48 (m, 2H, CH_2), 1.63-1.78 (m, 2H, CH_2), 2.30-2.56 (m, 1H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 13.8 (CH_3), 16.7 (CH_3), 20.3 (CH_2), 35.6 (CH_2), 39.1 (CH), 183.3 (CO). EI-MS (70 eV) (MSTFA derivative): *m/z* (%): 188 (1) $[M]^+$, 173 (61), 155 (5), 146 (15), 130 (6), 117 (7), 73 (100).

(*S*)-Methyl 2-methylpentanoate ((*S*)-62)

This reaction was performed according to the general procedure **H**.

Amounts used: 1.5 g (13 mmol) of the acid (***S***)**-61**, 25 ml of absolute methanol, 1.83 g (13 mmol) of borontrifluoride diethyletherate.

Yield: 1.58 g (93%).

R_f = 0.80 (pentane/diethyl ether 5:1). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 0.90 (t, J = 6.8 Hz, 3H, CH_3), 1.14 (d, J = 7.0 Hz, 3H, CH_3), 1.29 (m, 2H, CH_2), 1.41-1.60 (m, 2H, CH_2), 2.25-2.55 (m, 1H, CH), 3.67 (s, 3H, OCH_3). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 13.2 (CH_3), 16.3 (CH_3), 17.7 (CH_2), 35.3 (CH_2), 38.5 (CH), 50.7 (OCH_3), 176.7 (CO). EI-MS (70 eV): *m/z* (%): 131 (1) $[M]^+$, 115 (3), 101 (17), 88 (100), 71 (23), 57 (18), 43 (40).

(*S*)-2-Methylpentan-1-ol ((*S*)-63)

This reaction was performed according to the general procedure **I**.

Amounts used: 1.58 g (12 mmol) of the ester (***S***)**-62**, 50 ml of anhydrous diethyl ether, 1.83 g (48 mmol) of lithium aluminium hydride.

Yield: 1.2 g (98%).

R_f = 0.16 (pentane/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.90 (t, J=6.8 Hz, 3H, CH_3), 1.21 (d, J = 7.0 Hz, 3H, CH_3), 1.33-1.41 (m, 2H, CH_2), 1.63-1.78 (m, 2H, CH_2), 2.30-2.56 (m, 1H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 13.6 (CH_3), 15.9 (CH_3), 19.5 (CH_2), 35.3 (CH_2), 38.5 (CH), 182.9 (CO). EI-MS (70 eV) (MSTFA derivative): *m/z* (%): 174 (1) $[M]^+$, 159 (100), 103 (66), 89 (7), 75 (93), 73 (74).

(*S*)-1-Bromo-2-methylpentane ((S)-64)

Triphenylphosphine (3 g, 11.7 mmol) was dissolved in 25 ml of absolute dichloromethane and was cooled to 0°C. Bromine (0.7 ml, 11.7 mmol) was added to the reaction mixture drop-wise at the same temperature followed by the addition of (*S*)-2-methylpentan-1-ol ((***S***)-**63**) (1.2 g, 11.7 mmol). The reaction mixture was stirred overnight.[109] The work up was carried out by the addition of sodium hydrogen carbonate leading to the separation of two phases. The organic phase was separated and the aqueous phase was extracted two times with dichloromethane. All the organic phases were combined, dried with magnesium sulfate, filtered and concentrated in vacuo. Crude product was purified by column chromatography using pentane as solvent to get the final pure product **(S)-64**.

Yield: 1.4 g (72%).

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.91 (t, J = 6.8 Hz, 3H, CH_3), 1.01 (d, J =7.0 Hz, 3H, CH_3), 1.19-1.32 (m, 2H, CH_2), 1.34-1.39 (m, 2H, CH_2), 1.82 (m, 1 H, CH), 3.37 (m, 2H, CH_2Br). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 14.1 (CH_3), 18.7 (CH_3), 20.0 (CH_2), 37.0 (CH_2), 37.9 (CH) 41.5 (CH_2Br). EI-MS (70 eV): *m/z* (%): 164 (2) $[M]^+$, 121 (9), 85 (100), 71 (21), 55 (19), 43 (85).

(*S*)-4-Methylheptan-1-ol ((*S*)-65)

Preparation of Grignard reagent

Magnesium turnings (0.203 g, 8.5 mmol) were added to a dry round bottomed flask placed under inert atmosphere. Absolute tetrahydrofuran was added to the magnesium turnings such that only the surface of the magnesium was covered with the solvent. Later 2 drops of 1,2-dibromoethane was added to the reaction mixture (in order to activate the magnesium surface). Now a few drops of (*S*)-1-bromo-2-methylpentane ((***S***)-**64**) were added to this reaction mixture until bubbling of the reaction mixture was observed (if the bubbling was not observed for a long time the reaction flask can be heated with a heat gun). Stirring was started at this point, followed by the addition of remaining (*S*)-1-bromo-2-methylpentane ((***S***)-**64**) (1.4 g, 8.5 mmol) dissolved in 2.5ml of absolute tetrahydrofuran and the reaction was cooled with a water reflux for about 45 minutes. Completion of the reaction was confirmed

only when no heat was felt at the bottom of the flask. The reaction mixture was diluted with 12.5 ml of absolute tetrahydrofuran.

Coupling reaction with Li_2CuCl_4

To a stirred solution of 2-bromoethanol (0.425 g, 3.4 mmol) in 6 ml of absolute tetrahydrofuran, lithium tetrachloro cuprate (2.0 ml, 0.1*M* in THF) was added and cooled to 0°C under inert atmosphere. To this reaction mixture the above prepared solution of the Grignard reagent was added and allowed to stir at 0°C for 45 minutes. Later the ice bath was removed and the reaction mixture was stirred for 3-4 hours. Workup was carried out by hydrolyzing with concentrated hydrochloric acid leading to separation of the two phases.[110] The aqueous phase was extracted with diethyl ether, separated, and the organic phase was once washed with sodium hydrogen carbonate and once with sodium chloride. The separated aqueous phase was extracted with diethyl ether (x2). All the organic phases were combined, dried over magnesium sulphate, filtered, evaporated under vacuum and the crude product was purified by column chromatography using 20:1 pentane/diethyl ether mixture to get the final pure product **(*S*)-65**.

Yield: 1. 0 g (90%).

R_f = 0.06 (pentane/diethyl ether 20:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.85 (t, *J* = 6.8 Hz, 3H, CH_3), 1.01 (d, *J* = 7.0 Hz, 3H, CH_3), 1.16-1.21 (m, 2H, CH_2), 1.26-1.33 (m, 2H, CH_2), 1.36-1.43 (m, 1H, CH), 1.45-1.54 (m, 2H, CH_2), 1.58-1.63 (m, 2H, CH_2), 3.62 (t, *J* = 6.6 Hz, 2H, CH_2OH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 14.3 (CH_3), 19.5 (CH_3), 20.0 (CH), 30.3 (CH_2), 32.3 (CH), 32.9 (CH_2), 39.2 (CH_2), 63.4 (CH_2OH). EI-MS (70 eV): *m/z* (%): 129 (1) $[M]^+$, 112 (3), 97 (4), 84 (67), 69 (100), 56 (66), 41 (89).

(*S*)-4-Methylheptanoic acid ((*S*)-66)

***In situ* generation of (*S*)-4-methylheptanal**[111]

This reaction was performed according to the general procedure **J**.

Amounts used: 1.0 g (7.6 mmol) of the alcohol (***S*)-65** in 10 ml anhydrous dichloromethane, DMSO (1.43 g, 18.4 mmol) in 4 ml anhydrous dichloromethane,

oxalylchloride (1.0 g, 8.61 mmol) in 16 ml absolute dichloromethane, triethylamine (3.5 g, 35.3 mmol).

The resulting crude product was directly transformed into its corresponding acid ((***S***)-**66**) without any further purification.

Yield: 0.6 g (61%).

EI-MS (70 eV): *m/z* (%): 128 (1) $[M]^+$, 110 (3), 95 (15), 84 (46), 69 (26), 56 (100), 43 (85).

Oxidation of (*S*)-4-methylheptanal[112]

To a solution of (*S*)-4-methylheptanal (0.6 g, 4.6 mmol) in ethanol (87.6 ml), silver nitrate (0.985 mg, 6 mmol) dissolved in water (11.5 ml) was added which gave a homogeneous solution. Sodium hydroxide (775 mg, 19 mmol) in water (37.5 ml) was added with stirring over a period of 30 minutes. After stirring for further 3 h, the mixture was filtered, acidified with hydrochloric acid, and washed with diethyl ether. The organic phase was separated, the aqueous phase was extracted two times with diethyl ether and the combined ether phases were dried over magnesium sulfate and concentrated to give a yellow colored compound. Purification was carried out by column chromatography using 3:1 pentane/diethyl ether solvent mixture.

Yield: 0.4 g (58%).

R_f = 0.69 (pentane/diethyl ether 3:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.86 (t, J = 6.8 Hz, 3H, CH_3), 0.89 (d, J = 7.0 Hz, 3H, CH_3), 1.23-1.28 (m, 2H, CH_2), 1.32 (q, J = 8.7 Hz, 2H, CH_2), 1.49 (m, 1H, CH), 1.63 (dt, J = 9.1 Hz, J = 8.2 Hz, 2H, CH_2), 2.3 (t, J = 7.0 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.2 (CH_3), 19.1 (CH_3), 19.9 (CH_2), 31.6 (CH_2), 31.8 (CH_2), 32.0 (CH), 38.8 (CH_2), 180.5 (CO). EI-MS (70 eV): *m/z* (%): 145 (43) $[M]^+$, 127 (15), 112 (29), 84 (61), 71 (53), 57 (58), 43 (100).

Heptadecyl (*S*)-4-methylheptanoate ((*S*)-50)

This reaction was performed according to the general procedure **G**.

Amounts used: 0.1 g (0.7 mmol) of the acid (***S***)**-66**, 6 ml of absolute dichloromethane, 0.177 g (0.7 mmol) of heptadecanol, 0.008 g (0.07 mmol) of DMAP, 0.148 g (0.771 mmol) of 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC).

Yield: 0.137 g (51%).

R_f = 0.72 (pentane/diethyl ether 10:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.86 (t, J = 6.8 Hz, 6H, 2xCH_3), 0.89 (d, J = 7.1 Hz, 3H, CH_3), 1.21-1.41 (m, 32H, 16xCH_2), 1.45 (m, 1H, CH), 1.60-1.65 (m, 4H, 2xCH_2), 2.28 (dd, J = 9.3 Hz, J = 7.9 Hz, 2H, CH_2CO), 4.05 (t, J = 6.4 Hz, 2H, CH_2COO). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.1 (CH_3), 14.2 (CH_3), 19.2 (CH_3), 20.0 (CH_2), 22.6 (CH_2), 25.9 (CH_2), 28.6 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.5 (2xCH_2), 29.6 (CH_2), 29.6 (5xCH_2), 31.9 (2xCH_2), 32.1 (CH_2), 32.2 (CH_2CO), 38.9 (CH_2), 64.4 (CH_2COO), 174.2 (CO). EI-MS (70 eV): *m/z* (%): 382 (2) $[M]^+$, 325 (3), 311 (5), 283 (3), 238 (8), 210 (3), 145 (100), 127 (19), 109 (20), 97 (32), 83 (44), 69 (32).

8.3.5 Synthesis of (*S*)-14-methylheptadecyl (*S*)-4-methylheptanoate ((*S*)-(*S*)-51)

(*S*)-14-methylheptadecan-1-ol ((*S*)-68)

Procedure:

The procedure followed to synthesize (*S*)-14-methylheptadecan-1-ol ((***S***)**-68**) was similar to the synthesis of ((***S***)**-65**) with following chemicals.

Chemicals for preparation of Grignard reagent

Magnesium turnings (0.76 g, 3.1 mmol), 2 drops of 1,2-dibromoethane, 0.527 g (3.1 mmol) of (*S*)-1-bromo-2-methylpentane ((**S**)**-64**), 3ml of absolute tetrahydrofuran for the dilution of the reaction mixture.

Chemicals for Coupling reaction with Li_2CuCl_4

0.338 g (12.7 mmol) of 12-bromododecan-1-ol (**67**) in 3 ml of dry tetrahydrofuran, lithium tetrachlorocuprate (0.5 ml, 0.1M in THF). The crude product was purified by

column chromatography using 3:1 pentane/diethyl ether mixture to get the final pure compound (***S***)-**68**.

Yield: 0.720 g (21%).

R_f = 0.26 (pentane/diethyl ether 3:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.84 (d, J = 6.8 Hz, 3H, CH_3), 0.88 (t, J = 7.0 Hz, 3H, CH_3), 1.11-1.39 (m, 28H, 14xCH_2), 1.41 (m, 1H, CH), 1.58 (quin, J = 6.7 Hz, 2H, CH_2), 3.4 (t, J = 7.1, 2H, CH_2OH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.4 (CH_3), 19.6 (CH_3), 20.1 (CH_2), 25.7 (CH_2), 27.0 (CH_2), 29.4 (CH_2), 29.6 (CH_2), 29.7 (2xCH_2), 29.8 (2xCH_2), 29.9 (CH_2), 30.0 (CH_2), 32.4 (CH), 32.8 (CH_2), 37.0 (CH_2), 39.4 (CH_2), 63.0 (CH_2OH). EI-MS (70 eV): *m/z* (%): 269 (1) $[M]^+$, 252 (1), 224 (2), 209 (4), 181 (3), 168 (2), 153 (4), 139 (7), 125 (14), 111 (26), 97 (56), 84 (62), 69 (64), 55 (84), 43 (100).

(*S*)-14-methylheptadecyl (*S*)-4-methylheptanoate ((*S*)-(*S*)-51)

This reaction was performed according to the general procedure **G**.

Amounts used: 0.1 g (0.7 mmol) of the acid (***S***)**-66**, 6 ml of absolute dichloromethane, 0.189 g (0.7 mmol) of (*S*)-14-methylheptadecan-1-ol ((***S***)-**68**, 0.008 g (0.07 mmol) of DMAP, 0.148 g (0.771 mmol) of 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC).

Yield: 0.09 g (32%).

R_f = 0.72 (pentane/diethyl ether 10:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.84 (t, J = 6.8 Hz, 3H, CH_3), 0.86 (d, J = 7.0 Hz, 3H, CH_3), 0.89 (t, J = 7.1 Hz, 3H, CH_3), 0.90 (d, J = 6.8 Hz, 3H, CH_3), 1.19-1.33 (m, 30H, 15x CH_2), 1.36-1.50 (m, 2H, 2xCH), 1.62 (m, 4H, 2x CH_2), 2.30 (dd, J = 9.8 Hz, J = 8.2 Hz, 2H, CH_2CO), 4.07 (t, J = 6.7 Hz, 2H, CH_2COO). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.2 (CH_3), 14.4 (CH_3), 19.2 (CH_3), 19.6 (CH_3), 20.0 (CH_2), 20.1 (CH_2), 25.9 (CH_2), 27.0 (CH_2), 28.6 (CH_2), 29.2 (2xCH_2), 29.5 (2xCH_2), 29.6 (2xCH_2), 29.7 (CH_2), 30.0 (CH_2), 31.9 (CH_2), 32.1 (CH), 32.2 (CH), 32.4 (CH_2), 37.0 (CH_2), 38.9 (CH_2), 39.4 (CH_2), 64.4 (CH_2COO), 174.2 (CO). EI-MS (70 eV): *m/z* (%): 396 (6) $[M]^+$, 339 (6), 325 (9), 297 (4), 253 (4), 209 (5), 145 (100), 127 (24), 109 (26), 97 (30), 83 (35), 69 (32), 57 (42), 43 (73).

8.3.6 Synthesis of tricosa-2,4-diene (79)

Methyl nonadecanoate (96)

This reaction was performed according to the general procedure **H**.

Amounts used: 1 g (3.3 mmol) of the acid **95**, 25 ml of absolute methanol, 0.48 g (3.3 mmol) of boron trifluoride diethyl etherate.

Yield: 1.0 g (97%).

R_f = 0.76 (pentane/diethyl ether 5:1). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.89 (t, J = 6.80 Hz, 3H, CH_3), 1.19-1.32 (m, 30H, 15x CH_2), 1.60 (quin, J = 7.1 Hz, 2H, CH_2), 2.30 (t, J = 7.4 Hz, 2H, CH_2CO), 3.68 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.1 (CH_3), 22.6 (CH_2), 24.9 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (8xCH_2), 31.9 (CH_2), 34.1 (CH_2CO), 51.4 (OCH_3), 174.3 (CO). EI-MS (70 eV): *m/z* (%): 312 (34) $[M]^+$, 281 (10), 269 (28), 255 (6), 227 (7), 213 (11), 199 (8), 185 (5), 143 (27), 129 (7), 101 (11), 87 (63), 74 (100), 43(80).

Nonadecan-1-ol (97)

This reaction was performed according to the general procedure **I**.

Amounts used: 1.0 g (3.2 mmol) of the ester **96**, 25 ml of anhydrous diethyl ether, 0.480 g (13 mmol) of lithium aluminium hydride.

Yield: 0.870 g (96%).

R_f = 0.12 (pentane/diethyl ether 5:1). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.88 (t, J = 6.4 Hz, 3H, CH_3), 1.26-1.34 (m, 32H, 16xCH_2), 1.57 (quin, J = 7.7 Hz, 2H, CH_2CH_2OH), 3.64 (t, J = 6.6, 2H, CH_2OH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.1 (CH_3), 22.6 (CH_2), 25.7 (CH_2), 29.3 (CH_2), 29.4 (2xCH_2), 29.5 (2xCH_2), 29.6 (8xCH_2), 31.9 (CH_2), 32.8 (CH_2CH_2OH), 63.1 (CH_2OH). EI-MS (70 eV) (MSTFA derivative): *m/z* (%): 356 (1) $[M]^+$, 341 (100), 325 (3), 129 (9), 115 (14), 103 (11), 75 (22), 57 (19), 43 (26).

Nonadecanal (98)

This reaction was performed according to the general procedure **J**.

Amounts used: 0.87 g (3.0 mmol) of the alcohol **97** in 5 ml anhydrous dichloromethane, DMSO (0.8 g, 7.6 mmol) in 2 ml anhydrous dichloromethane, oxalylchloride (0.466 g, 3.6 mmol) in 8 ml anhydrous dichloromethane, triethylamine (1.4 g, 13.7 mmol).

Yield: 0.6 g (61%).

R_f = 0.60 (pentane/diethyl ether 5:1). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.88 (t, J = 6.4 Hz, 3H, CH_3), 1.21-1.45 (m, 30H, 15x CH_2), 1.63 (tt, J = 7.3 Hz, J = 6.6 Hz, 2H, CH_2CH_2CO), 2.42 (t, J = 7.2 Hz, 2H, CH_2COH), 9.76 (s, 1H, COH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.0 (CH_3), 22.0 (CH_2CH_2COH), 22.6 (CH_2), 29.1 (CH_2), 29.20 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.56 (8xCH_2), 31.9 (CH_2), 43.9 (CH_2COH), 202.0 (CO). EI-MS (70 eV): *m/z* (%): 281 (1) $[M]^+$, 264 (3), 236 (3), 152 (2), 138 (5), 137 (6), 124 (9), 110 (14), 96 (48), 82 (75), 57 (80), 43 (100).

(*E*)-But-2-en-1-yltriphenylphosphonium bromide (100)

This reaction was performed according to the general procedure **K**.

Amounts used: 1.3 g (9.6 mmol) crotyl bromide **99**, 2.52 (9.6 mmol) triphenyl phosphine, 80 ml of anhydrous toluene.

Yield: 2.84 g (93%).

^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.59-1.64 (m, 3H, CH_3), 4.69 (dd, J = 14.5 Hz, J = 7.2 Hz, 2H, CH_2), 5.37 (dq, J = 14.2 Hz, J = 7.3 Hz, 1H, CH), 5.93-6.05 (m, 1H, CH), 7.65-7.69 (m, 6H, CH), 7.70-7.79 (m, 3H, CH), 7.80-7.92 (m, 6H, CH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 18.3 (CH_3), 28.4 (CH_2), 114.8 (CH), 118.6 (3xCH), 130.3 (6xCH), 133.9 (6xCH), 135.0 (3xCH), 137.8 (CH).

Tricosa-2,4-diene (79)

This reaction was performed according to the general procedure **L**.

Amounts used: 0.456 g (1.43 mmol) of Wittig salt **100** dissolved in 25 ml of anhydrous dimethoxy ethane, 5 ml of Li-HMDS (1M in hexane) 0.410 g (1.43 mmol) of the aldehyde **98** dissolved in 2 ml of DME.

Yield: 0.225 g (49%).

R_f = 0.87 (pentane/diethyl ether 10:1). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.88 (t, J = 6.63 Hz, 3H, CH_3), 1.18-1.45 (m, 32H, 16xCH_2), 1.65 (m, 3H, CH_3), 2.05 (q, J = 8.2 Hz, 2H, CH_2), 5.49-5.59 (m, 1H, CH), 5.56-5.72 (m, 1H, CH), 5.90-5.99 (m, 1H, CH), 6.00-6.07 (m, 1H, CH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ:14.1 (CH_3), 18.2 (CH_3), 22.7 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (2xCH_2), 29.7 (8xCH_2), 31.9 (CH_2), 32.5 (CH_2), 127.0 (CH), 128.9 (CH), 130.2 (CH), 132.3 (CH). EI-MS (70 eV): *m/z* (%): 320 (20) $[M]^+$, 278 (3) 123 (5), 109 (9), 95 (22), 81 (65), 68 (100).

8.3.7 Synthesis of pentacosa-2,4-diene (80)

1-Iodoicodsane (102)

This reaction was performed according to the general procedure **M**.

Amounts used: 0.545 g (8 mmol) of imidazole dissolved in 30 ml of anhydrous acetonitrile/diethyl ether mixture (ratio 3:1), 2.1 g (8 mmol) of triphenyl phosphine, 2.1 g (8 mmol) of iodine, 1.25 g (4 mmol) of the alcohol **101** dissolved in 3 ml of anhydrous diethyl ether.

Yield: 0.134 g (41%).

R_f = 0.92 (pentane/diethyl ether 10:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.88 (t, J = 6.6 Hz, 3H, CH_3), 1.19-1.32 (m, 32H, 16xCH_2), 1.35-1.41 (m, 2H, CH_2), 1.82 (quin, J = 7.2 Hz, 2H, CH_2CH_2I), 3.19 (t, J = 7.0 Hz, 2H, CH_2I). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 7.2 (CH_2I), 14.1 (CH_3), 22.6 (CH_2), 28.5 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (2xCH_2), 29.7 (8xCH_2), 30.5 (CH_2), 31.9 (CH_2), 33.6 (CH_2CH_2I). EI-MS (70 eV):

m/z (%): 407 (1) $[M]^+$, 281 (11), 197 (6), 183 (5), 169 (5), 155(11),141 (7), 127 (11),113 (14), 99 (22), 85 (57), 71 (76), 57 (100).

Henicosanenitrile (103)

This reaction was performed according to the general procedure **N**.

Amounts used: 1.0 g (6.5 mmol) of tetraethyl ammoniumcyanide dissolved in 15 ml of DMSO, 1.34 g (3.2 mmol) of the iodide **102** dissolved in 20 ml of DMSO.

Yield: 1.0 g (100%).

R_f = 0.50 (pentane/diethyl ether 10:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.89 (t, *J* = 6.6 Hz, 3H, CH_3), 1.19-1.32 (m, 32H, 16xCH_2), 1.35-1.41 (m, 2H, CH_2), 1.82 (quin, *J* = 7.2 Hz, 2H, CH_2CH_2CN), 2.33 (t, *J* = 7.0 Hz, 2H, CH_2CN). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 14.1 (CH_3), 17.2 (CH_2CN), 22.6 (CH_2), 25.5 (CH_2CH_2CN), 29.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (8xCH_2), 29.7 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 32.0 (CH_2), 119.8 (CN). EI-MS (70 eV): *m/z* (%): 307 (1) $[M]^+$, 278 (14), 264 (23), 250 (19), 236 (18), 222 (15), 208 (14),194 (12), 180 (10), 166 (10), 152 (11), 138 (15), 124 (24), 110 (36), 97 (53), 57 (93), 43 (100).

Henicosanoic acid (104)

This reaction was performed according to the general procedure **O**.

Amounts used: 1.0 g (3.2 mmol) of cyanide **103** dissolved in 60% aqueous ethanol (35 ml), 2.79 g (68.4 mmol) of sodium hydroxide.

Yield: 1.0 g (95%).

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.89 (t, *J* = 6.6 Hz, 3H, CH_3), 1.19-1.32 (m, 32H, 16x CH_2), 1.35-1.41 (m, 2H, CH_2), 1.82 (quin, *J* = 7.2 Hz, 2H, CH_2CH_2COOH), 2.35 (t, *J* = 7.6 Hz, 2H, CH_2COOH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 14.1 (CH_3), 22.6 (CH_2), 25.1 (CH_2CH_2COOH), 29.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (8xCH_2), 29.7 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 32.0 (CH_2), 34.2 (CH_2COOH), 178.9 (COOH). EI-MS (70 eV) (MSTFA derivative): *m/z* (%): 398 (11) $[M]^+$, 383 (100), 339 (6), 255 (4), 145 (31), 129 (45), 117 (69), 73 (83).

Methyl henicosanoate (105)

This reaction was performed according to the general procedure **H**.

Amounts used: 1 g (3.0 mmol) of the acid **104**, 25 ml of absolute methanol, 0.44 g (3.0 mmol) of boron trifluoride diethyl etherate.

Yield: 0.82 g (80%).

R_f = 0.76 (pentane/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.89 (t, J = 6.4 Hz, 3H, CH_3), 1.19-1.32 (m, 32H, 16xCH_2), 1.35-1.41 (m, 2H, CH_2), 1.62 (quin, J = 7.4 Hz, 2H, CH_2CH_2CO), 2.30 (t, J = 7.4 Hz, 2H, CH_2CO), 3.66 (s, 3H, OCH_3). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 14.1 (CH_3), 22.7 (CH_2), 25.0 (CH_2CH_2CO), 29.1 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (2xCH_2), 29.5 (2xCH_2), 29.7 (8xCH_2), 31.9 (CH_2), 34.1 (CH_2CO), 51.4 (OCH_3), 174.3 (COOMe). EI-MS (70 eV): *m/z* (%): 340 (21) $[M]^+$, 325 (2), 297 (14), 255 (6), 241 (7), 199 (7), 185 (5), 143(19), 129 (7), 87 (70), 74 (100),

Henicosan-1-ol (106)

This reaction was performed according to the general procedure **I**.

Amounts used: 0.82 g (2.4 mmol) of the ester **105**, 25 ml of anhydrous diethyl ether, 0.361 g (9.6 mmol) of lithium aluminium hydride.

Yield: 0.760 g (100%).

R_f = 0.17 (pentane/diethyl ether 10:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: = 0.89 (t, J = 6.4 Hz, 3H, CH_3), 1.19-1.32 (m, 34H, 17xCH_2), 1.35-1.41 (m, 2H, CH_2), 1.54 (quin, J = 7.0 Hz, 2H, CH_2CH_2OH), 3.66 (t, J = 7.4 Hz, 3H, CH_2OH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 14.1 (CH_3), 22.7 (CH_2), 25.8 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (2xCH_2), 29.5 (2xCH_2), 29.7 (8xCH_2), 31.7 (CH_2), 32.9 (CH_2CH_2OH), 63.4 (CH_2OH). EI-MS (70 eV) (MSTFA derivative): *m/z* (%): 384 (1) $[M]^+$, 369 (100), 207 (5), 103 (19), 75 (42).

Henicosanal (107)

This reaction was performed according to the general procedure **J**.

Amounts used: 0.6 g (1.9 mmol) of the alcohol **106** in 3 ml absolute dichloromethane, Dimethylsulphoxide (0.375g, 4.8 mmol) in 2 ml absolute dichloromethane, oxalylchloride (0.292 g, 2.3 mmol) in 5 ml absolute dichloromethane, triethylamine (0.88 g, 8.6 mmol).

Yield: 0.41 g (69%).

R_f = 0.65 (pentane/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.89 (t, J = 6.4 Hz, 3H, CH_3), 1.19-1.32 (m, 32H, 16xCH_2), 1.35-1.41 (m, 2H, CH_2), 1.61 (quin, J = 7.3 Hz, 2H, CH_2CH_2CO), 2.46 (t, J = 7.4 Hz, 2H, CH_2CO), 10.1 (s, 1H, CHO). ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 14.1 (CH_3), 21.9 (CH_2CH_2CHO), 22.7 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (2xCH_2), 29.5 (2xCH_2), 29.7 (8xCH_2), 31.7 (CH_2), 43.9 (CH_2CHO), 202.9 (CHO). EI-MS (70 eV): *m/z* (%): 310 (1) $[M]^+$, 292 (7), 264 (5), 236 (4), 208 (4), 180 (4), 152 (4), 138 (9), 124 (13), 110 (19), 96 (59), 82 (80), 57 (86), 43 (100).

Pentacosa-2,4-diene (80)

This reaction was performed according to the general procedure **L**.

Amounts used: 0.456 g (1.43 mmol) of Wittig salt **100** dissolved in 25 ml of anhydrous dimethoxy ethane, 5 ml of Li-HMDS (1M in hexane) 0.447 g (1.43 mmol) of the aldehyde **107** dissolved in 2 ml of DME.

Yield: 0.210 g (42%).

R_f = 0.88 (pentane/diethyl ether 10:1). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.88 (t, J = 6.8 Hz, 3H, CH_3), 1.21-1.38 (m, 36H, 18xCH_2), 1.65 (m, 3H, CH_3), 2.05 (q, J = 8.2 Hz, 2H, CH_2), 5.49-5.59 (m, 1H, CH), 5.56-5.72 (m, 1H, CH), 5.90-5.99 (m, 1H, CH), 6.00-6.07 (m, 1H, CH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.1 (CH_3), 18.3 (CH_3), 22.7 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (2xCH_2), 29.7 (10xCH_2), 31.9 (CH_2), 32.5 (CH_2), 127.0 (CH), 128.9 (CH), 130.1 (CH), 132.3 (CH). EI-MS (70

eV): *m/z* (%): 348 (14) $[M]^+$, 306 (3), 137 (4), 123 (6), 110 (10), 96 (25), 81 (65), 68 (100).

8.3.8 Synthesis of methyl-(11*Z*,14*Z*,17*Z*) 2,2,3,3-tetradeuteroicosa-11,14,17,19-tetraenoate (114)

7-Oxabicyclo[4.1.0]hept-3-ene (125)

To a stirred solution of 1,4 cyclohexadiene (**124**) (3.5 g, 42 mmol) in dichloromethane (8 ml) at -5°C was added slowly a solution of *m*-CPBA (70% in water, 10.2 g, 41.3 mmol) and K_2HPO_4 in dichloromethane (300 ml). The resulting reaction mixture was stirred for 18 h at room temperature followed by successive washings with saturated sodium hydrogen carbonate, 5% sodium sulfate, water and saturated sodium chloride.[165] The separated aqueous phase was extracted with dichloromethane (x2) and the combined organic solvents were dried over magnesium sulfate, filtered, evaporated under *vacuo* yielding crude product which was purified on silica gel by using 5:1 pentane/diethyl ether as eluent mixture.

Yield: 3.9 g (97%).

R_f = 0.30 (pentane/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 2.50-2.54 (m, *2*H, 2xCH_2), 3.25-3.29 (m, 2H, 2xCHO), 5.45-5.55 (m, 2H, 2xCH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 24.3 (2xCH_2), 50.4 (2xCHO), 120.9 (2xCH). EI-MS (70 eV): *m/z* (%): 96 (41) $[M]^+$, 81 (10), 77 (9), 68 (53), 67 (100), 65 (26), 53 (21), 41 (45).

(*Z*)-Hex-3-ene-1,6-diol (126)

To a solution of epoxide **125** (1.9 g, 19.7 mmol) in a 1:1 THF/H_2O mixture, was added 10% sulfuric acid (3 ml) and the resulting reaction mixture was refluxed for 30 minutes. Then, the reaction was cooled to 10°C, followed by the addition of $NaIO_4$ (4.7 g, 22 mmol) over 15 minutes. After stirring the reaction mixture for 1 h at that temperature, neutralization (pH 7.0-7.5) with saturated sodium hydrogen carbonate was followed by the addition of $NaBH_4$ (1.16 g, 30.6 mmol) in water (2 ml) over 1 h period of time to the neutralized mixture. Stirring was continued at 10°C for further 30 minutes. After the successive additions of acetone (8 ml) and saturated sodium

chloride (125 ml) the reaction mixture was extracted with ethyl acetate (x3). Combined organic extracts were washed successively with saturated sodium hydrogen sulfite, water, 5% HCl, saturated sodium hydrogen carbonate, and saturated sodium chloride, dried over magnesium sulfate, and filtered.[166] Solvent evaporation in *vacuo*, followed by column chromatography on silica gel with 10:1 methanol/diethyl ether as eluent mixture yielded pure product **126**.

Yield: 1.24 g (54%).

R_f = 0.54 (methanol/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 2.28-2.40 (m, 4H, 2xCH_2), 3.64 (t, J = 6.0 Hz, 4H, 2xCH_2), 5.51-5.60 (m, 2H, 2xCH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 32.1 (2xCH_2), 61.4 (2xCH_2OH), 126.8 (2xCH). EI-MS (70 eV): *m/z* (%): 98 (9) $[M-18]^+$, 83 (4), 69 (11), 68 (100), 55 (60), 41 (37).

(*Z*)-1,6-Diiodohex-3-ene (127)

A suspension of triphenylphosphine (5.2 g, 20 mmol) and imidazole (1.4 g, 20 mmol) in a mixture of diethyl ether and acetonitrile (3:1, 40 ml) was treated at 0°C with iodine (5 g, 20 mmol). After being stirred for 10 min at room temperature, the suspension was re-cooled to 0°C and (*Z*)-hex-3-ene-1,6-diol (**126**) (1.04 g, 9 mmol) in ether (4 ml) was added. After additional stirring for 3 h at room temperature, the reaction mixture was poured into saturated $NaHCO_3$.[167] Extraction with pentane (x3), drying over magnesium sulfate and evaporation of the solvent gave crude (*Z*)-1,6-diiodo-hex-3-ene (**127**) which was used in generation of **122** without further purification.

Yield: 3.0 g (99%)

(*Z*)-Hex-3-enyl-1,6-bis(triphenylphosphonium iodide) (122)

Crude product of **127** (3.0 g, 8.9 mmol) was transferred into a solution of triphenylphosphine (5.8g, 11 mmol) in acetonitrile (100 ml). After refluxing for 4h the mixture was poured into toluene (1 L) and the resulting Wittig salt was filtered off.[167] Recrystallization from methanol/diethyl ether afforded white colored pure crystals of **122**.

Yield: 4.21 g (52%).

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 2.26-2.39 (m, 4H, 2xCH_2), 3.45-3.54 (m, 4H, 2x CH_2P), 5.89-6.09 (m, 2H, 2xCH), 7.69-7.81 (m, 24H, 24xCH), 7.86-7.90 (m, 6H, 6xCH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 20.9 (2xCH_2P), 22.7 (2xCH_2), 118.7 (6xC), 121.9 (2xCH), 129.8 (12xCH), 133.7 (12xCH), 134.1 (6xCH).

Methyl undec-10-enoate (129)

This reaction was performed according to the general procedure **H**.

Amounts used: 10 g (54 mmol) of the acid **128**, 60 ml of absolute methanol, 7.6 g (54 mmol) of borontrifluoride diethyletherate.

Yield: 10.1 g (94%).

R_f = 0.76 (pentane/diethyl ether 3:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.19-1.43 (m, 10H, 5xCH_2), 1.61 (quin, J = 7.1 Hz, 2H, CH_2CH_2CO), 1.99-2.08 (m, 2H, CH_2CH) 2.30 (t, J = 7.1 Hz, 2H, CH_2CO), 3.67 (s, 3H, OCH_3), 4.89-4.98 (m, 1H, CH), 5.01-5.05 (m, 1H, CH), 5.71-5.88 (m, 1H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 24.9 (CH_2CH_2CO), 28.8 (CH_2), 29.0 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 33.7 (CH_2CH), 34.0 (CH_2CO), 51.3 (OCH_3), 114.1 (CH), 139.1 (CH), 174.2 (CO). EI-MS (70 eV): *m/z* (%): 198 (1) $[M]^+$, 166 (15), 149 (15), 137 (11), 124 (41), 110 (12), 96 (45), 95 (13), 87 (61), 74 (100), 69 (40), 55 (95), 41 (68).

Methyl 2,2-dideuteroundec-10-enoate (130)

This reaction was performed according to the general procedure **P**.

Amounts used: 17.4 g (757 mmol) of the sodium metal, 90 ml of $[D_1]$-methanol, 10 g (50 mmol) of the ester **129** dissolved in 10 ml of $[D_1]$-methanol.

Yield: 2.3 g (23%).

R_f = 0.48 (pentane/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.19-1.43 (m, 10H, 5xCH_2), 1.61 (quin, J = 7.1 Hz, 2H, CH_2CD_2CO), 1.99-2.08 (m, 2H, CH_2CH),

3.67 (s, 3H, OCH_3), 4.89-4.98 (m, 1H, CH), 5.01-5.05 (m, 1H, CH), 5.71-5.88 (m, 1H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 24.8 (CH_2CH_2CO), 28.8 (CH_2), 29.0 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 33.7 (CH_2CH), 34.7 (CD_2CO), 51.4 (OCH_3), 114.1 (CH), 139.1 (CH), 174.2 (CO). EI-MS (70 eV): *m/z* (%): 200 (1) $[M]^+$, 168 (16), 151 (15), 139 (11), 124 (31), 112 (13), 98 (52), 88 (38), 76 (100), 69 (40), 55 (86), 41 (84).

1,1,2,2-Tetradeuteroundec-10-en-1ol (131)

This reaction was performed according to the general procedure **I**.

Amounts used: 2 g (10 mmol) of the deuterated ester **130**, 25 ml of anhydrous diethyl ether, 1.67 g (40 mmol) of lithium aluminium deuteride.

Yield: 1.7 g (98%).

R_f = 0.16 (pentane/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.19-1.43 (m, 12H, 6xCH_2), 1.99-2.08 (m, 2H, CH_2CH), 4.89-4.98 (m, 1H, CH), 5.01-5.05 (m, 1H, CH), 5.71-5.88 (m, 1H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 25.4 (CH_2CD_2), 28.9 (CH_2), 29.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 31.7 (CD_2CD_2OH), 33.7 (CH_2CH), 65.8(CD_2OH), 114.1 (CH), 139.1 (CH). EI-MS (70 eV): *m/z* (%): 156 (2) $[M-18]^+$, 124 (8), 113 (15), 99 (39), 85 (41), 81 (53), 72 (46), 68 (66), 55 (92), 41 (100).

1,1,2,2-Tetradeutero-1-iodo-undec-10-en (132)

This reaction was performed according to the general procedure **M**.

Amounts used: 1.63 g (9.36 mmol) of the deuterated alcohol **131**, 4.9 g (18.7 mmol) of triphenyl phosphine, 1.28 g (18.7 mmol) of imidazole, 3:1 diethyl ether/acetonitrile mixture (40 ml), 4.74g (18.7 mmol) of iodine.

Yield: 2.0 g (76%).

R_f = 0.86 (pentane/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.19-1.43 (m, 12H, 6xCH_2), 1.99-2.08 (m, 2H, CH_2CH), 4.89-4.98 (m, 1H, CH), 5.01-5.05 (m, 1H, CH), 5.71-5.88 (m, 1H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 2.6 (CD_2I), 28.4 (CH_2), 28.8 (CH_2), 29.0 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 30.2 (CH_2CD_2), 33.7 (CH_2CH),

34.1(CD_2CD_2I), 114.1 (CH), 139.1 (CH). EI-MS (70 eV): *m/z* (%): 284 (6) $[M]^+$, 242 (22), 228 (2), 200 (5), 158 (15), 143 (3), 127 (2), 114 (8), 100 (28), 83 (50), 69 (52), 55 (100), 41 (70).

1,1,2,2-Tetradeuteroundec-10-en carbonitrile (133)

This reaction was performed according to the general procedure **N**.

Amounts used: 2.0 g (12.9 mmol) of tetraethyl ammonium cyanide dissolved in 25 ml of DMSO, 1.85 g (6.5 mmol) of the iodide **132** dissolved in 20 ml of DMSO.

Yield: 1.2 g (100%).

R_f = 0.45 (pentane/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.19-1.47 (m, 12H, 6xCH_2), 1.99-2.08 (m, 2H, CH_2CH), 4.89-4.98 (m, 1H, CH), 5.01-5.05 (m, 1H, CH), 5.71-5.88 (m, 1H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 15.2 (CD_2CN), 24.4 (CD_2CD_2CN), 28.3 (CH_2CD_2), 28.6 (CH_2), 28.8 (CH_2), 29.0 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 33.7 (CH_2CH), 114.1 (CH), 119.8 (CN), 139.1 (CH). EI-MS (70 eV): *m/z* (%): 182 (6) $[M-1]^+$, 168 (6), 154 (26), 150 (6), 140 (76), 126 (100), 100 (26), 82 (21), 69 (32), 55 (67), 41 (71).

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2,2,3,3-Tetradeuterododec-11-enoic acid (134)

This reaction was performed according to the general procedure **O**.

Amounts used: 1.2 g (6.5 mmol) of cyanide **133** dissolved in 60% [D_1]-ethanol (70 ml) (dissolved in D_2O), 5.4 g (135 mmol) of NaOD.

Yield: 0.76 g (58%).

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.19-1.47 (m, 12H, 6xCH_2), 1.99-2.08 (m, 2H, CH_2CH), 4.89-4.98 (m, 1H, CH), 5.01-5.05 (m, 1H, CH), 5.71-5.88 (m, 1H, CH). ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 24.4 (CD_2CD_2CO), 28.3 (CH_2CD_2), 28.6 (CH_2), 28.8 (CH_2), 29.0 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 33.7 (CH_2CH), 35.1 (CD_2CO), 114.1 (CH), 139.1 (CH), 179.2 (CO). EI-MS (70 eV) (MSTFA derivative): *m/z* (%): 274 (1) $[M]^+$, 259 (100), 217 (4), 202 (3), 184 (8), 148 (12), 132 (40), 119 (75), 73 (72).

Methyl 2,2,3,3-tetradeuterododec-11-enoate (135)

This reaction was performed according to the general procedure **G**.

Amounts used: 0.7 g (3.5 mmol) of the acid **134**, 25 ml of anhydrous methanol, 0.042 g (0.35 mmol) DMAP, 0.8 g (4.1 mmol) of 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC).

Yield: 0.6 g (80%).

R_f = 0.75 (pentane/diethyl ether 5:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.19-1.47 (m, 12H, 6xCH_2), 1.99-2.08 (m, 2H, CH_2CH), 3.67 (s, 3H, CH_3), 4.89-4.98 (m, 1H, CH), 5.01-5.05 (m, 1H, CH), 5.71-5.88 (m, 1H, CH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 24.2 (CD_2CD_2CO), 28.8 (CH_2CD_2), 28.9 (CH_2), 29.0 (CH_2), 29.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 33.3 (CD_2CO), 33.7 (CH_2CH), 51.4 (OCH_3), 114.1 (CH), 139.2 (CH), 174.3 (CO). EI-MS (70 eV) *m/z* (%): 216 (1) $[M]^+$, 184 (19), 166 (5), 155 (6), 141 (20), 140 (26), 127 (15), 114 (14), 100 (37), 90 (40), 76 (100), 55 (46).

Methyl 2,2,3,3-tetradeutero-11-oxoundecanoate(123)

Preparation of $RuCl_3$ stock solution

To a solution of H_2O (5 ml) $RuCl_3$ (0.042 g, 0.2 mmol) was added and mixed well which gave the stock solution that was stable at room temperature for several months.

Oxidation of the double bond

To a solution of methyl 2,2,3,3-tetradeuterododec-11-enoate **(135)** (0.6 g, 2.7 mmol) in 6:1 mixture of acetonitrile/H_2O (28 ml), was added $RuCl_3$ stock solution (2.8 ml) followed by the addition of $NaIO_4$ (1.2 g, 5.6 mmol) in portions over a period of 5 minutes at room temperature. The reaction was monitored by TLC for 2 h after which the completed reaction mixture was quenched with saturated solution of sodium thiosulfate.[171] The separated aqueous phase was extracted with ethyl acetate(x3). Combined organic phases were washed with water and saturated sodium chloride, dried over magnesium sulfate, filtered and concentrated in *vacuo* to give the crude product that was purified on silica gel using 1:1 mixture of hexane/ethyl acetate.

Yield: 0.48 g (81%).

R_f = 0.75 (hexane/ethyl acetate 1:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.21 (t, J = 7.0 Hz, 2H, CH_2CD_2), 1.26-1.34 (m, 8H, 4xCH_2), 1.62 (tt, 2H, J = 7.1 Hz, J = 6.9 Hz, CH_2CH_2CHO), 2.42 (dt, J = 9.8 Hz, J = 7.1 Hz, CH_2CHO) 3.67 (s, 3H, CH_3), 9.76 (t, J = 2.1 Hz, 1H, CHO). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 22.0 (CH_2CH_2CHO), 24.8 (CD_2CD_2COOMe), 28.8 (CH_2CD_2), 28.9 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 35.0 (CD_2COOMe), 43.8 (CH_2CHO), 51.4 (OCH_3), 174.9 (COOMe), 202.8 (CHO). EI-MS (70 eV) *m/z* (%): 219 (1) $[M]^+$, 190 (14), 175 (38), 168 (6), 143 (47), 125 (11), 115 (11), 101 (19), 90 (38), 76 (100), 59 (28), 41 (28).

Methyl-(11*Z*,14*Z*,17*Z*) 2,2,3,3-tetradeuteroicosa-11,14,17,19-tetraenoate (120)

Generation of bis(ylide) 136

A cold (-78°C) suspension of the Wittig salt **122** (0.878 g, 1 mmol) in anhydrous THF (20 ml) was treated with Li-HMDS (2.2 ml of 1.0M solution in hexane, 1.1 mmol). The reaction mixture was allowed to warm to room temperature, stirred for 30 min and re-cooled (-78 °C).[167]

Procedure for reductive olefination

An etheral solution of the aluminate was prepared as follows: To a cold (-78°C) solution of the respective methyl ester **123** (0.219 g, 1 mmol) in anhydrous diethyl ether (5 ml) was added drop-wise a pre-cooled (-78°C) DIBAL-H (1 ml of 1M solution in hexane, 1 mmol). After being stirred for 60 minutes the cold (-78°C) aluminate was transferred quickly to the bis(ylide) **136** reaction mixture with a cannula. The mixture was allowed to warm to room temperature over a period of 90 minutes, and stirring was continued for another 1 h before re-cooling to -78°C. Then, a freshly distilled solution of the second aldehyde component acrolein (**121**) (0.067 g, 1.2 mmol) dissolved in anhydrous THF (1 ml) was added. The mixture was allowed to reach room temperature and stirred for 30 more minutes. Hydrolysis with HCl (2 N), extraction with diethyl ether, drying over magnesium sulfate, filtration followed by evaporation under *vacuo* afforded crude product. The product **120** was obtained only in minute quantity, purification of which proved difficult from the mixture containing undesired homo conjugated by-products.

Yield: ≤1% (GC value).

EI-MS (70 eV) *m/z* (%): 322 (23) $[M]^+$, 291 (3), 161 (6), 147 (16), 133 (35), 119 (36), 105 (39), 93 (80), 91 (100), 79 (97), 67 (63), 55 (25), 41 (35).

8.3.9 Synthesis of methyl-(11*Z*,14*Z*,17*Z*) 3,3,4,4-tetradeuteroicosa-11,14,17-trienoate (142)

Methyl- (9*Z*,12*Z*,15*Z*) octadeca-9,12,15-trienoate (138)

This reaction was performed according to the general procedure **H**.

Amounts used: 1 g (3.5 mmol) of the acid **137**, 20 ml of absolute methanol, 0.51 g (3.5 mmol) of boron trifluoride diethyl etherate.

Yield: 1.0 g (98%).

R_f = 0.50 (pentane/diethyl ether 2:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.97 (t, *J* = 7.4 Hz, 3H, CH_3), 1.29-1.32 (m, 4H, 2xCH_2), 1.33-1.35 (m, 4H, 2xCH_2), 1.59 (quin, *J* = 7.7 Hz, 2H, CH_2CH_2CO), 2.03-2.08 (m, 4H, 2xCH_2), 2.26 (t, J = 7.1 Hz, 2H, CH_2CO), 2.79-2.82 (m, 2H, 2xCH_2), 3.63 (s, 3H, CH_3), 5.28-5.43 (m, 6H, 6xCH). EI-MS (70 eV) *m/z* (%): 292 (21) $[M]^+$, 261 (8), 236 (10), 173 (8), 149 (25), 135 (30), 121 (36), 108 (57), 95 (61), 79 (100), 79 (97), 67 (59), 55 (43).

Methyl- (9*Z*,12*Z*,15*Z*) 2,2-dideuterooctadeca-9,12,15-trienoate (139)

This reaction was performed according to the general procedure **P**.

Amounts used: 1.2 g (52.2 mmol) of the sodium metal, 50 ml of $[D_1]$-methanol, 1.0 g (3.42 mmol) of the ester **138** dissolved in 5 ml of $[D_1]$-methanol.

Yield: 0.71 g (71%).

R_f = 0.89 (pentane/diethyl ether 5:1). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.97 (t, *J* = 7.4 Hz, 3H, CH_3), 1.29-1.32 (m, 4H, 2xCH_2), 1.33-1.35 (m, 4H, 2xCH_2), 1.57 (t, *J* = 7.7 Hz, 2H, CH_2CD_2CO), 2.03-2.08 (m, 4H, 2xCH_2), 2.79-2.82 (m, 2H, 2xCH_2), 3.63 (s, 3H, CH_3), 5.28-5.43 (m, 6H, 6xCH). EI-MS (70 eV) *m/z* (%): = 294 (11) $[M]^+$, 263 (6),

238 (7), 163 (4), 149 (13), 135 (15), 121 (20), 108 (41), 95 (51), 79 (100), 67 (59), 55 (28), 41 (35).

(9*Z*,12*Z*,15*Z*)-1,1,2,2-Tetradeuterooctadeca-9,12,15-trien-1-ol (140)

This reaction was performed according to the general procedure **I**.

Amounts used: 3.23 g (11.2 mmol) of the deuterated ester **139**, 60 ml of anhydrous diethyl ether, 1.88 g (44.8 mmol) of lithium aluminium deuteride.

Yield: 2.98 g (99%).

R_f = 0.45 (pentane/diethyl ether 1:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.98 (t, J = 7.6 Hz, 3H, CH_3), 1.29-1.32 (m, 4H, 2xCH_2), 1.33-1.35 (m, 6H, 3xCH_2), 2.03–2.08 (m, 4H, 2xCH_2), 2.79-2.82 (m, 4H, 2xCH_2), 5.28-5.43 (m, 6H, 6xCH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.3 (CH_3), 20.5 (CH_2), 25.5 (CH_2), 25.6 (CH_2), 26.2 (CH_2CD_2), 27.2 (CH2), 29.2 (CH2), 29.3 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 127.1 (CH), 127.7 (CH), 128.3 (2xCH), 130.3 (CH), 131.9 (CH). EI-MS (70 eV) *m/z* (%): = 268 (8) $[M]^+$, 212 (11), 149 (5), 135 (11), 121 (14), 108 (45), 95 (31), 93 (49), 79 (100), 67 (52), 55 (23), 41 (29).

(9*Z*,12*Z*,15*Z*)-1,1,2,2-Tetradeutero-1-iodo-octadeca-9,12,15-triene (141)

This reaction was performed according to the general procedure **M**.

Amounts used: 2.98 g (11.1 mmol) of the deuterated alcohol **140**, 5.8 g (22.2 mmol) of triphenylphosphine, 1.51g (22.2 mmol) of imidazole, 3:1 diethyl ether/acetonitrile mixture (80 ml), 5.7g (22.2 mmol) of iodine.

Yield: 3.5 g (84%).

R_f = 0.93 (pentane/diethyl ether 5:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.98 (t, J = 7.5 Hz, 3H, CH_3), 1.29-1.32 (m, 4H, 2xCH_2), 1.33-1.35 (m, 6H, 3xCH_2), 2.03-2.08 (m, 4H, 2xCH_2), 2.81 (t, J = 5.7 Hz, 4H, 2xCH_2), 5.28-5.43 (m, 6H, 6xCH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.3 (CH_3), 20.6 (CH_2), 25.5 (CH_2), 25.6 (CH_2), 27.2 (CH_2), 28.5 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.6 (CH_2), 30.3 (CH_2), 127.1 (CH), 127.7 (CH), 128.3

(2xCH), 130.3 (CH), 132.0 (CH). EI-MS (70 eV) *m/z* (%): = 378 (11) $[M]^+$, 322 (18), 159 (4), 149 (3), 135 (7), 121 (11), 108 (67), 93 (40), 79 (100), 67 (58), 55 (28), 41 (33).

(11*Z*,14*Z*,17*Z*)-Methyl 3,3,4,4-tetradeuteroicosa-11,14,17-trienoate (142)

To a solution of diisopropylamine (3.04 g, 30 mmol) in dry THF (12 ml) at -78°C was added *n*-BuLi (12.5 ml, 1.6M in hexane) drop-wise. The resulting mixture was stirred for further 10 minutes before diluting the mixture with anhydrous THF (15 ml). To this solution, methyl acetate (0.686 g, 9.25 mmol) dissolved in anhydrous THF (2 ml) was added and allowed to stir at -78°C for 30 more minutes. Then tetradeuterated iodide **141** (1.75 g, 4.62 mmol) was added and stirring was continued at -78°C for 4 h followed by raising it to room temperature in 1 h.[73] The reaction mixture was treated with HCl (2M) and extracted with diethyl ether (x3). The combined organic phases were washed with sodium hydrogen carbonate and dried over magnesium sulfate. Filtration, evaporation of solvent under *vacuo* followed by purification of the crude product on silica gel column with 5:1 pentane/diethyl ether mixture as eluent gave pure product **142**.

Yield: 0.18 g (12%).

R_f = 0.72 (pentane/diethyl ether 5:1). EI-MS (70 eV) *m/z* (%): = 324 (11) $[M]^+$, 293 (11), 268 (10), 149 (12), 135 (23), 121 (31), 108 (51), 95 (60), 79 (100), 67 (58), 55 (26), 41 (31).

8.3.10 Synthesis of (11*Z*,14*Z*,17*Z*)-2,2,3,3,4,4-hexadeuteroicosa-11,14,17-trienoic acid (144)

(10*Z*,13*Z*,16*Z*)-2,2,3,3-Tetradeuterononadeca-10,13,16-triene-1- carbonitrile(143)

Anhydrous acetonitrile (0.475 g, 11.5 mmol) dissolved in dry THF (12 ml) at -78°C was treated with *n*-BuLi (5 ml, 1.6M solution in hexane) under nitrogen atmosphere. The resulting reaction mixture was stirred at this temperature for 2h, followed by the addition of tetradeuterated iodide **141** (1.75 g, 4.62 mmol) dissolved in anhydrous THF (15 ml).[169] Stirring was continued at this temperature for 3 h, after which the

mixture was warmed to room temperature and stirred overnight. The mixture was hydrolyzed with H_2O and extracted with diethyl ether. The organic phase was separated and the aqueous phase was washed twice with diethyl ether. The combined organic phase was dried over magnesium sulfate, and the solvents were removed under reduced pressure. The crude product was purified by column chromatography on silica gel using 10:1 pentane/diethyl ether as eluent mixture

Yield: 0.41 g (30%).

R_f = 0.27 (pentane/diethyl ether 10:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.98 (t, J = 7.5 Hz, 3H, CH_3), 1.29-1.32 (m, 4H, 2xCH_2), 1.33-1.35 (m, 6H, 3xCH_2), 2.03- 2.08 (m, 4H, 2xCH_2), 2.38 (s, 2H, CH_2CN), 2.81 (t, J = 5.7 Hz, 4H, 2xCH_2), 5.27-5.42 (m, 6H, 6xCH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.3 (CH_3), 17.7 (CH_2CN), 20.6 (CH_2), 25.5 (CH_2), 25.6 (CH_2), 27.2 (CH_2), 28.5 (CH_2), 28.7 (CH2), 29.2 (CH_2), 29.3 (CH_2), 29.6 (CH_2), 119.8 (CN), 127.1 (CH), 127.7 (CH), 128.2 (CH), 128.3 (CH), 130.3 (CH), 131.9 (CH). EI-MS (70eV): *m/z* (%) = 291 (2) $[M]^+$, 262 (2), 248 (2), 235 (2), 222 (6), 180 (2), 166 (3), 149 (5), 135 (9), 121 (12), 108 (24), 95 (53), 79 (100), 67 (73), 55 (41), 41 (61).

(11*Z*,14*Z*,17*Z*)-2,2,3,3,4,4-hexadeuteroicosa-11,14,17-trienoic acid (144)

This reaction was performed according to the general procedure **O**.

Amounts used: 0.41 g (1.40 mmol) of cyanide **143** dissolved in 60% [D_1]-ethanol (20 ml) (dissolved D_2O), 1.1 g (28 mmol) of NaOD.

Yield: 0.195 g (45%).

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.97 (t, J = 7.6 Hz, 3H) 1.16 (t, J = 7.0 Hz, 2H, CH_2CD_2) 1.26-1.41 (m, 8H, 4xCH) 2.01-2.18 (m, 4H, 2xCH_2) 2.81 (t, J = 5.6, 4H, 2xCH_2) 5.27-5.42 (m, 6H, 6xCH). EI-MS (70eV) (MSTFA derivative): *m/z* (%) = 384 (16) $[M]^+$, 369 (75), 355 (6), 341 (5), 328 (7), 311 (4), 249 (2), 191 (6), 177 (7), 163 (8), 149 (18), 132 (40), 121 (27), 108 (54), 95 (61), 76 (100), 67 (50), 55 (20), 41 (23).

9. Abbreviations

α	Alpha
abs	Absolute
amu	Atomic mass unit
β	Beta
CLSA	Closed loop stripping analysis
CNS	Central nervous system
δ	Delta
d	Doublet
d.r.	Diastereomeric ratio
DME	1,2-Dimethoxyethane
DIBAL-H	Diisobutylaluminium hydride
DMAP	4-Dimethylaminopyridine
DMSO	Dimethyl sulfoxide
EAD	Electro antennographic detection
EDC	1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride
EI	Electron impact
eV	Electron volt
FA	Farnesyl acetate
γ	Gamma
GC	Gas chromatography
HA	Hexadecyl acetate
HBA	(*R*)-3-Hydroxybutyric acid
HBBA	(*R*,*R*)-3-(3-Hydroxybutyryloxy)-butyric acid
HPLC	High performance liquid chromatography
HS	Head space
J	Coupling constant

K-HMDS	Potassium hexamethyldisilazane
LDA	Lithium diisopropylamide
Li-HMDS	Lithium hexamethyldisilazane
m	Multiplet
m-CPBA	Meta-chloroperbenzoic acid
MSTFA	(*N*-Methyl-*N*-trimethylsilyltrifluoroacetamide)
m/z	Mass-to-charge ratio
MS	Mass spectroscopy
MSD	Mass spectrometer detector
n-BuLi	n-Butyllithium
Na-HMDS	Sodium hexamethyldisilazane
NMR	Nuclear magnetic resonance
NIST	National institute of standards and technology
ω	Omega
q	Quartett
quin	Quintett
R_f	Retention factor
RI	Retention Index
s	Singlet
Sext	Sextet
t	Triplet
TBDMS	Tertiary-butyldimethylsilyl
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TMSH	Trimethylsulfonium hydroxide
UV	Ultraviolet

10. Literature

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11. Appendix

11.1 Mass spectra

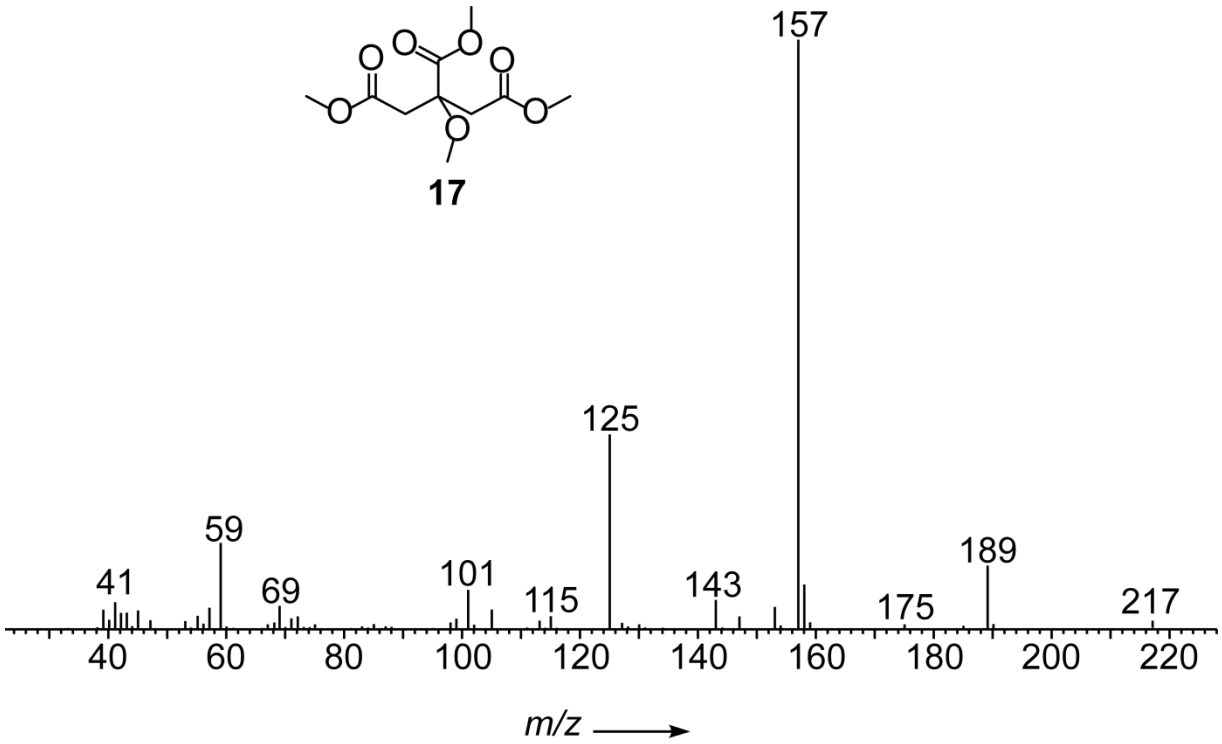

Figure 69. Mass spectrum of Trimethyl 2-methoxypropane-1,2,3-tricarboxylate (**17**)

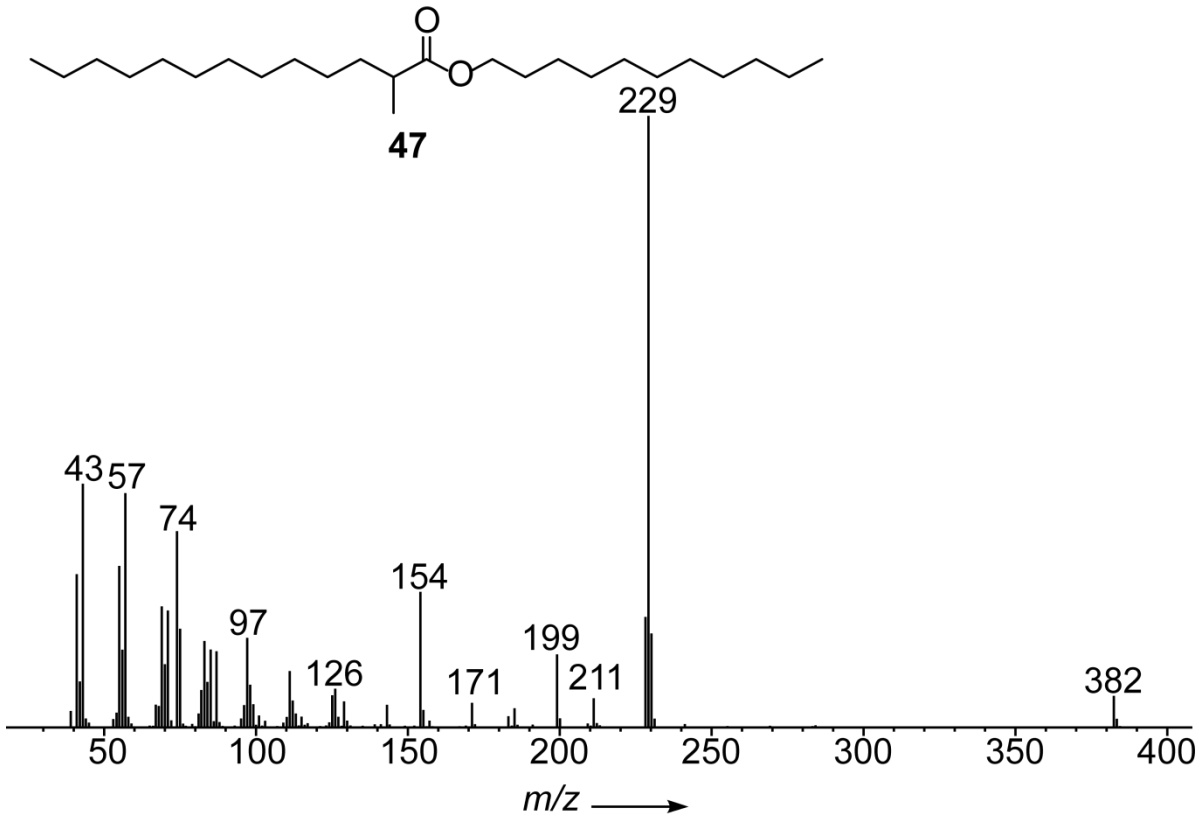

Figure 70. Mass spectrum of undecyl 2-methyltridecanoate (**47**)

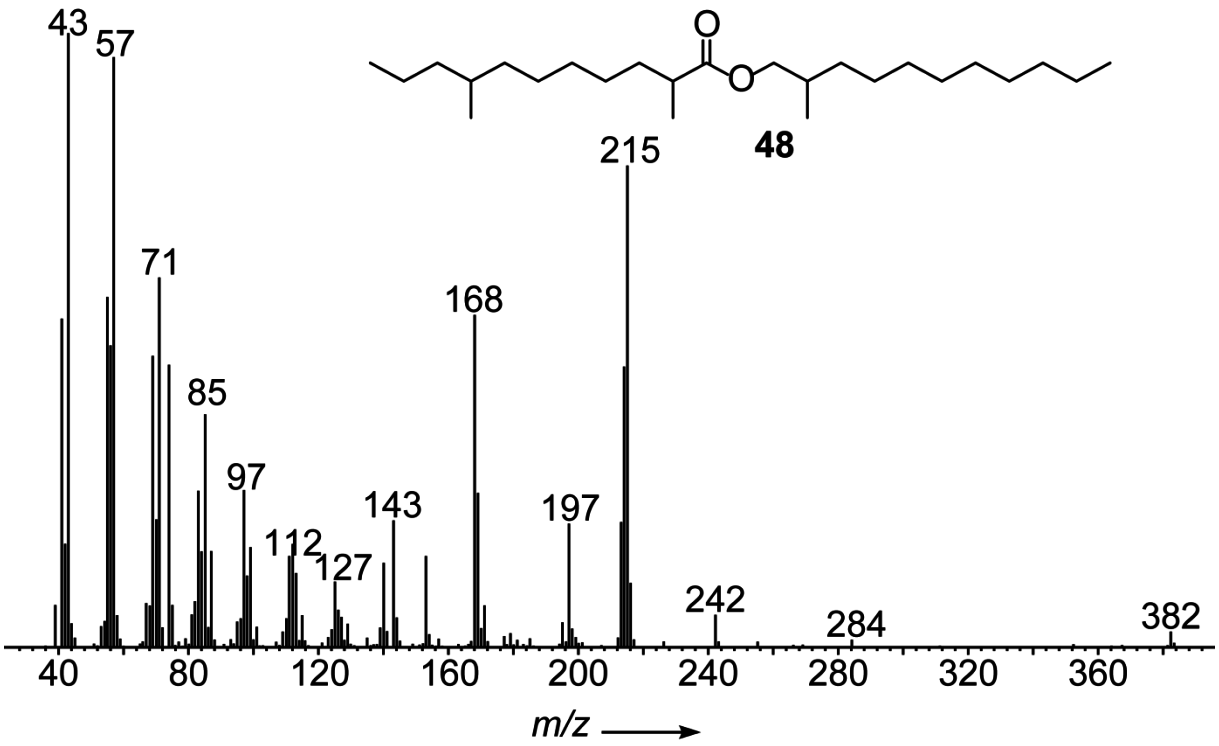

Figure 71. Mass spectrum of 2-methylundecyl 2,8-dimethylundecanoate (**48**)

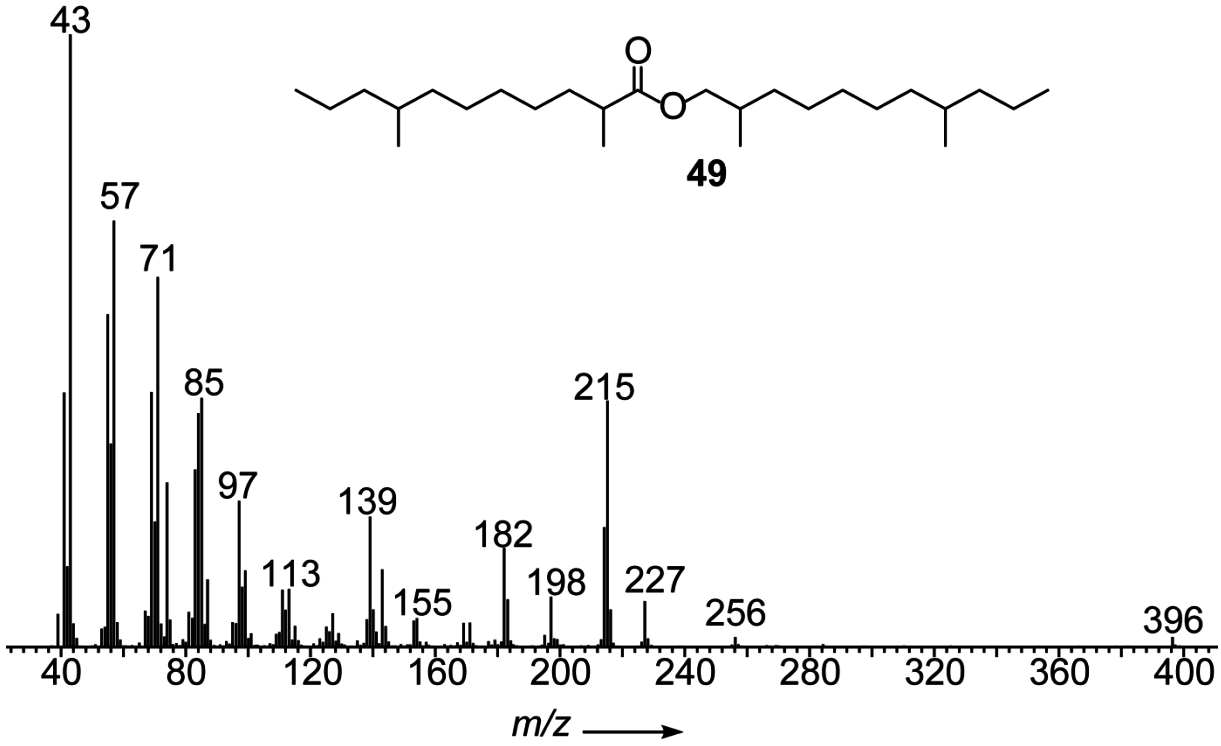

Figure 72. Mass spectrum of 2,8-dimethylundecyl 2,8-dimethylundecanoate (**49**)

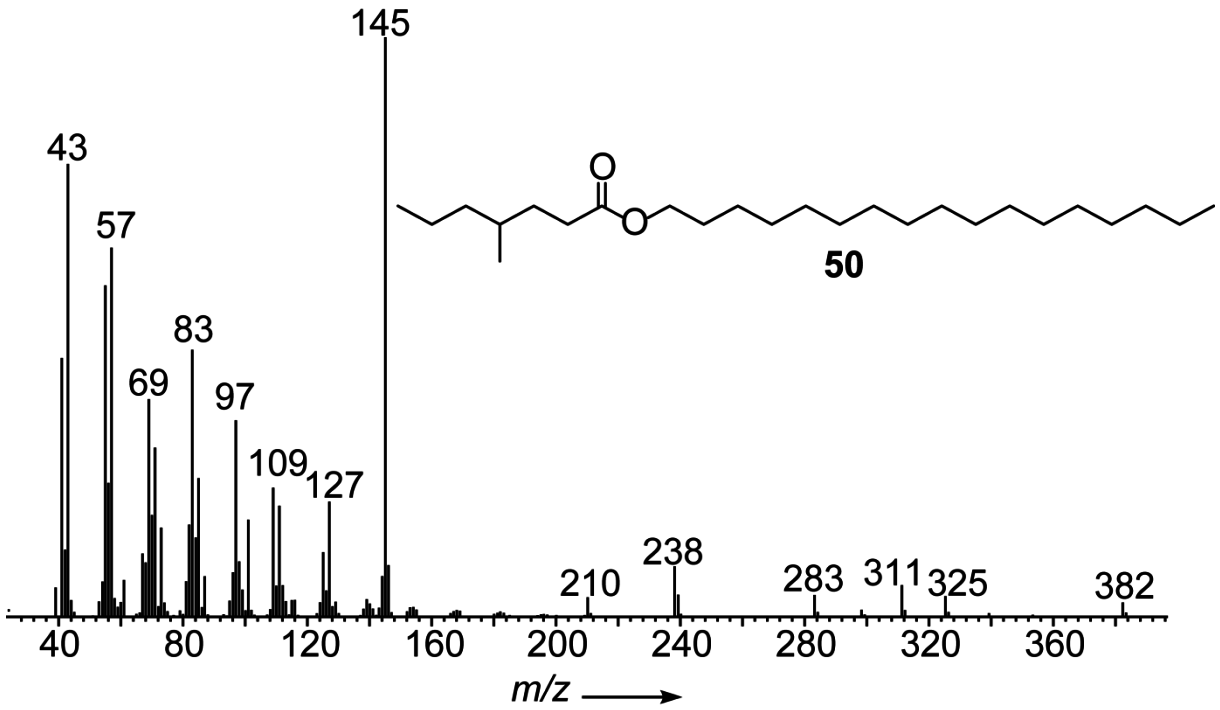

Figure 73. Mass spectrum of heptadecyl 4-methylheptanoate (**50**)

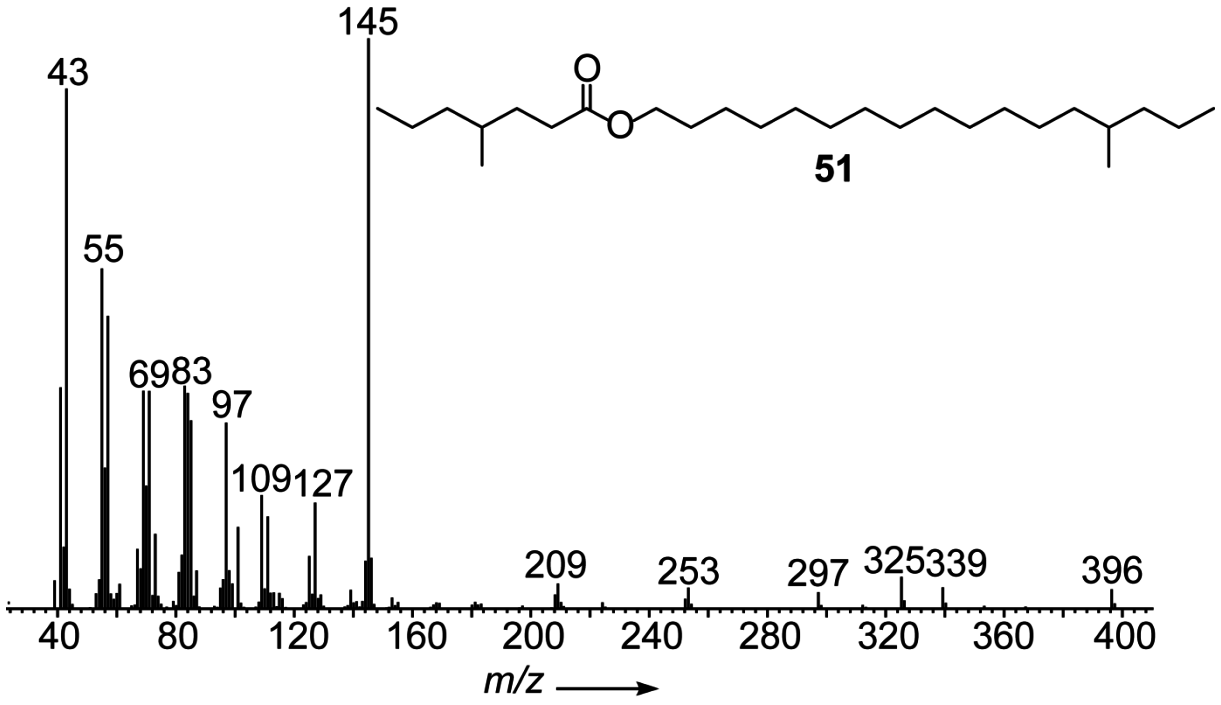

Figure 74. Mass spectrum of 14-methylheptadecyl 4-methylheptanoate (**51**)

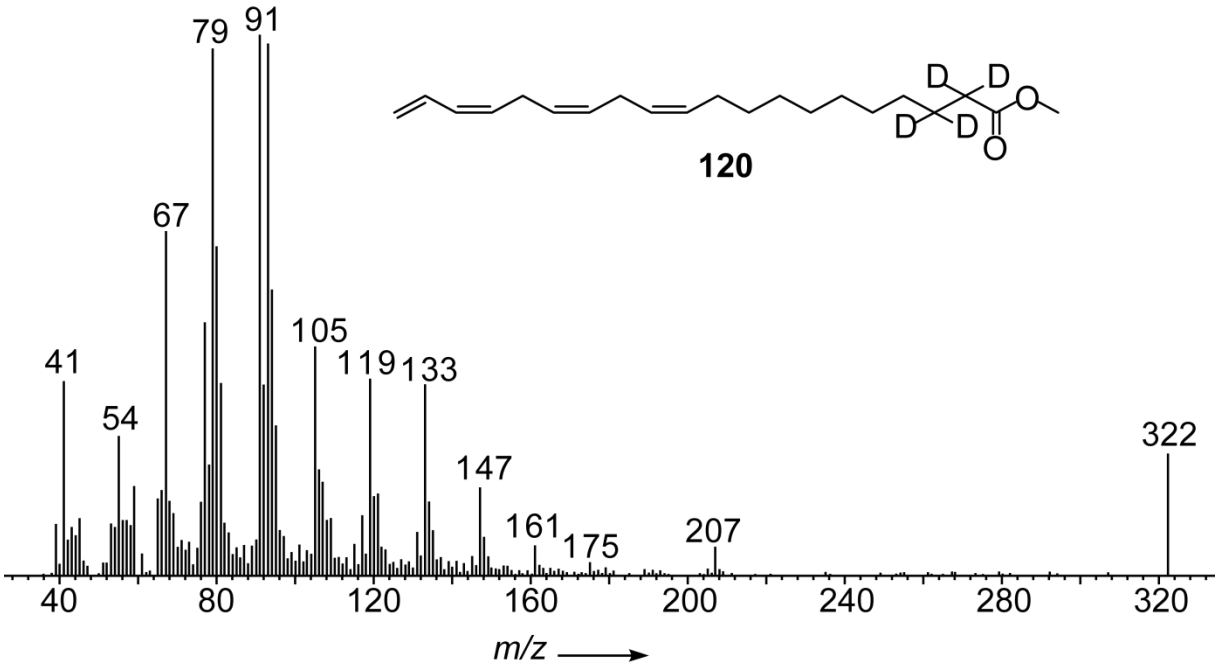

Figure 75. Mass spectrum of methyl-(11*Z*,14*Z*,17*Z*) 2,2,3,3-tetradeuteroicosa-11,14,17,19-tetraenoate (**120**)

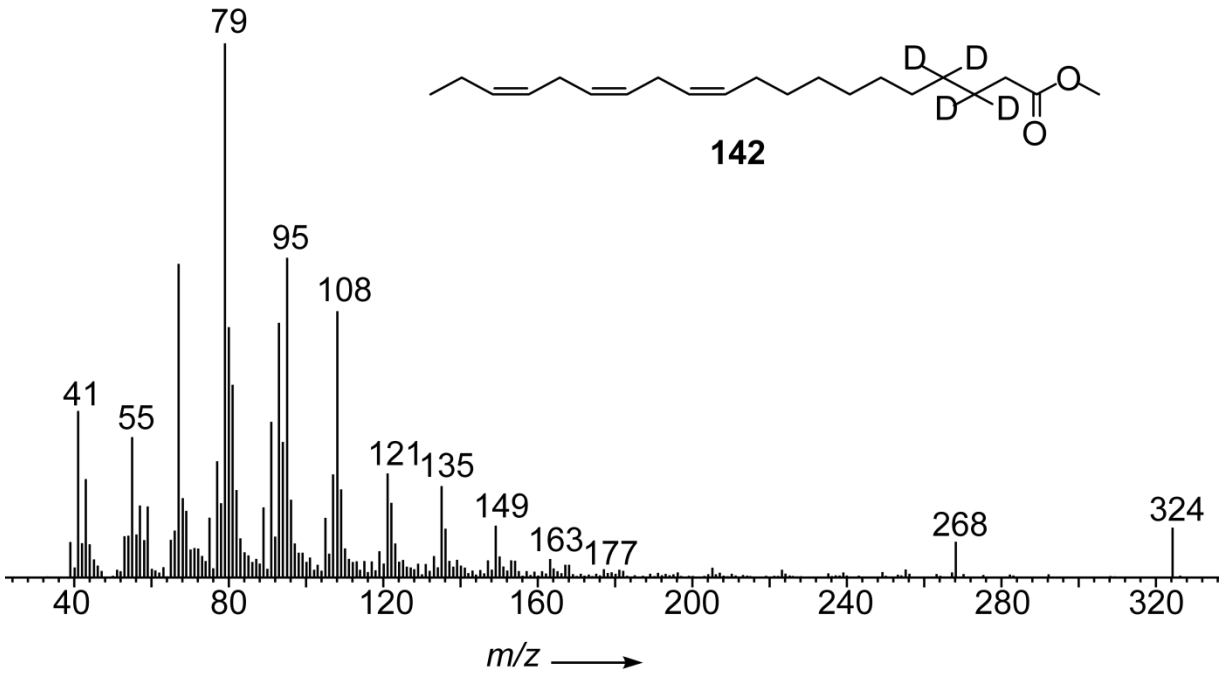

Figure 76. Mass spectrum of methyl-(11*Z*,14*Z*,17*Z*) 3,3,4,4-tetradeuteroicosa-11,14,17-trienoate (**142**)

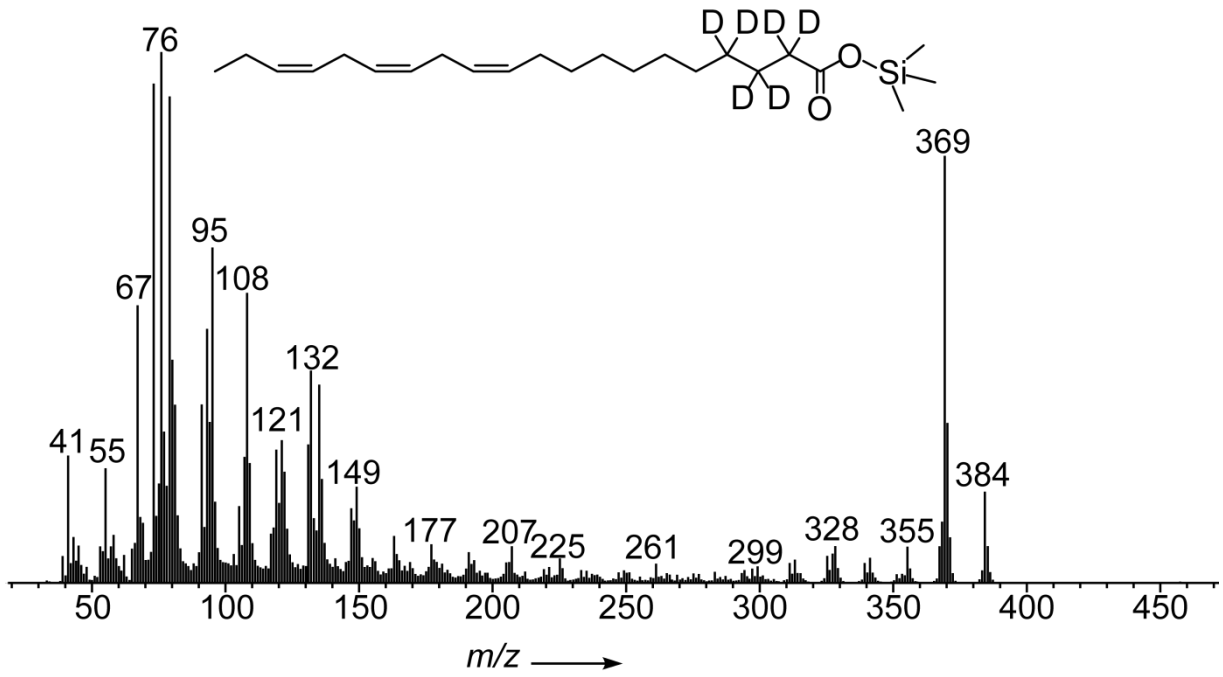

Figure 77. Mass spectrum of the corresponding trimethylsilyl ether of (11*Z*,14*Z*,17*Z*)-2,2,3,3,4,4-hexadeuteroicosa-11,14,17-trienoic acid (**144**)

Resume

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